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SHORT COMMUNICATION

p-Coumaric acid inhibition of CREB phosphorylation reduces cellular melanogenesis

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Abstract We investigated the in vitro effects of *p*-coumaric acid on melanogenesis. The melanin content in B16F1 cells stimulated with *p*-coumaric acid significantly decreased (68 % vs. control) through inhibition of tyrosinase enzyme activity assessed using both cell-free and cell-based assays (46 and 27 % compared with control, respectively). In addition, stimulating B16F1 cells with *p*-coumaric acid reduced cyclic adenosine monophosphate (cAMP)—responsive element-binding protein (CREB) protein phosphorylation (26 % vs. control), which in turn downregulated the expression of the microphthalmia-associated transcription factor (MITF) and its target gene tyrosinase (27 and 20 % vs. control, respectively). *p*-Coumaric acid has a hypopigmentation effect in melanocytes by both directly inhibiting tyrosinase enzyme activity and reducing CREB phosphorylation, which inhibits MITF and tyrosinase expression.

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Introduction

Cellular melanin concentration determines skin, hair, and eye color, and dysregulation of melanogenesis results in several medical problems including Addison's disease and cosmetic issues [1]. Therefore, the control of melanogenesis is important for skin health [2]. Melanogenesis is regulated by several exogenous (e.g., UV light, X-rays, and photo aging chemicals) and endogenous (e.g., hormones and cytokines) factors that have a complicated biosynthetic pathway including tyrosinase as a rate-limiting enzyme, which catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and subsequent oxidation of DOPA to dopaquinone [3]. Hence, inhibition of tyrosinase activity or downregulation of tyrosinase gene expression may be responsible for reduced melanin production. Tyrosinase is primarily regulated by the microphthalmia-associated transcription factor (MITF), a master transcription factor in melanogenesis [4]. MITF activation is positively regulated by the cAMP-dependent protein kinase A (PKA) signaling pathway; thus, increased cellular cAMP concentrations induce PKA activation, which phosphorylates and activates cAMP-responsive element-binding protein (CREB). This activation of CREB increases MITF gene expression and subsequently inhibits the transcription of MITF target genes, including tyrosinase [5].

Traditional depigmenting agents including hydroquinone and kojic acid remain highly effective; however, their use is associated with several safety concerns over long-term exposure (e.g., atrophy, carcinogenesis, ochronosis,

and other local or systemic side effects) [6]. Accordingly, identifying and characterizing natural anti-melanogenic compounds with minimal side effects is important.

Rice (*Oryza sativa* L.) bran has been used as a hypopigmenting agent in Korea, China, and Japan for hundreds of years. Rice bran contains a variety of well-known antioxidative and bioactive compounds; however, the primary active hypopigmenting compounds in rice bran are largely unknown, excluding ferulic acid, which shows a mild effect [7, 8]. We previously isolated 15 single compounds from rice bran and screened them for hypopigmenting activity. We found that *p*-coumaric acid significantly reduced cellular melanin content and its hypopigmentation effect was the most potent among 15 tested compounds; thus, we investigated its mechanism in cultured melanocytes. In this study, we examined the inhibitory effects of *p*-coumaric acid on melanogenesis. Moreover, the mechanism underlying the observed hypopigmentation was investigated in B16F1 melanoma cells.

Materials and methods

Materials

B16 melanocytes were purchased from the Korean Cell Line Bank (Seoul, South Korea). Purified *p*-coumaric acid was provided by the National Institute of Crop Science in Korea. In brief, brown rice (*Oryza sativa*, 1 kg) obtained from National Institute of Crop Science was extracted with MeOH (3X) at ambient temperature. After concentration of solvent, the water-based suspension was partitioned with *n*-hexane and EtOAc. Repeated column chromatofraphy (SiO_2) gave 23 mg of *p*-coumaric acid, identical in all respects including spectroscopic data to an authentic sample. Arbutin, mushroom tyrosinase, protease inhibitor cocktail, iQ SYBR Green Supermix, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA).

Cell culture, cellular melanin content, and Fontana–Masson staining

B16F1 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin/streptomycin at 37 °C under 5 % CO_2 [9]. Cellular melanin content was quantified according to a previously described method [10]. Fontana–Masson staining was performed with a commercially available kit (American MasterTech Scientific, Inc. Lodi, CA, USA) according to the manufacturer's instructions. Briefly, cells were stained with ammoniacal silver, gold chloride, and sodium thiosulfate.

Cell-free and cell-based tyrosinase activity assays

Cell-free and cell-based tyrosinase activity assays were performed according to our previous study [10].

Measurement of cAMP levels

Cellular cAMP levels in B16F1 melanoma cells treated with *p*-coumaric acid for 72 h were quantified using a colorimetric competitive immunoassay kit from Enzo Life Sciences (Farmingdale, NY, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) and immunoblotting analysis

qPCR was performed according to a method described previously [11] with the following primer sequences: MITF forward 5′-GTATGAACACGCACTCTCTC-3′ and reverse 5′-CTTCTGCGCTCATACTGCTC-3′; tyrosinase forward 5′-GGCCAGCTT TCAGGCAGAGGT-3′ and reverse 5′-TGGTGCTTCATGGGCAAA ATC-3′; cyclophilin forward 5′-GGCCGATGACGAGCCC-3′ and reverse 5′-TGT CTTTGGAACTTT-3′. Expression levels were calculated using the relative method, which compares the target and β-actin genes using iQ5 Optical System Software (version 2; Bio-Rad, Hercules, CA, USA). Immunoblotting analysis was performed according to a method described previously [11].

Statistical analysis

All data are expressed as the mean ± standard error. Values were compared using the Student's *t*-test. P values < 0.05 were considered statistically significant.

Results and discussion

p-Coumaric acid reduces cellular melanin content

We previously isolated 15 single compounds from rice bran and performed preliminary screening experiments by measuring cellular melanin content in cultured melanocytes. We found that *p*-coumaric acid was the most potently inhibited melanogenesis among tested rice compounds; thus, we examined the hypopigmentation mechanism of *p*-coumaric acid. Other studies have reported multiple *p*-coumaric acid bioactivities with no significant cytotoxicity in a cultured cell system [12], and we confirmed the nontoxicity using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Fontana–Masson staining showed a significant

reduction in cytosolic melanin as a brown-black pigment in *p*-coumaric acid-stimulated B16F1 melanoma cells compared to control cells (Fig. 1a). Arbutin was used as the positive control. Cellular melanin content decreased significantly by 68 % at 1 mM *p*-coumaric acid compared to control cells (Fig. 1b). These hypopigmenting activity is in line with previously published data confirming anti-melanogenic effects of *p*-coumaric acid [13].

p-Coumaric acid directly inhibits tyrosinase activity

Anti-melanogenic effects can be achieved either by directly inhibiting tyrosinase enzyme activity or by reducing melanogenic enzyme expression [14, 15]. *p*-Coumaric acid directly inhibited tyrosinase activity more potently than arbutin based on a cell-free, mushroom tyrosinase assay (Fig. 1b). *p*-Coumaric acid is nontoxic at 1 mM [16]; thus, we stimulated cells with a high concentration of *p*-coumaric acid. We also measured the level of tyrosinase activity in B16F1 melanoma cells stimulated with 1 mM *p*-coumaric acid for 72 h. *p*-Coumaric acid significantly reduced cellular tyrosinase activity by 27 % at 1 mM (Fig. 1b). The inhibitory effect of *p*-coumaric acid on cellular tyrosinase activity at 1 mM was comparable to that of arbutin. These indicate that *p*-coumaric acid could inhibit cellular melanin synthesis by directly inhibiting the rate-limiting enzyme activity in melanogenesis [14].

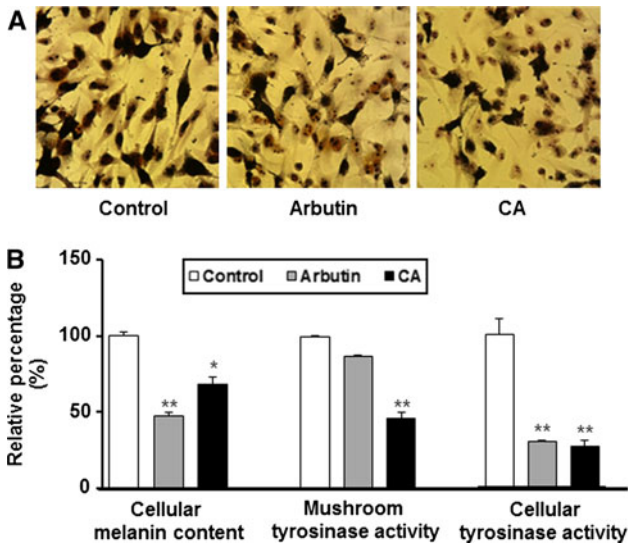

Fig. 1 Effect of *p*-coumaric acid on melanin synthesis and tyrosinase activity. **a** Melanin was stained brown using the Fontana–Masson staining method (1 mM *p*-coumaric acid). **b** Cellular melanin content and tyrosinase activity assay. Cellular tyrosinase activity was determined using B16F1 cell lysates. B16F1 cells were treated with *p*-coumaric acid or arbutin (positive control) for 72 h. $*P < 0.05$ compared to the control (no treatment). Data represent the mean ± SE of three independent experiments

p-Coumaric acid reduces CREB phosphorylation and MITF and tyrosinase expression

Melanogenesis could be inhibited by altered gene expressions, which can be mediated by cellular signaling pathway [17]. Accordingly, we next examined the effect of *p*-coumaric acid on the phosphorylation of PKA target signaling proteins and the expression of genes related to melanogenesis. The activation of PKA phosphorylates CREB, particularly on serine 133, and is a major stimulatory signal for MITF transcription [18]. Phosphorylated CREB (p-CREB) binds the cAMP-responsive element domain present in the MITF promoter and stimulates MITF gene expression. MITF then self-induces and promotes melanogenesis target gene transcription [19]. AKT and GSK are also activated by phosphorylation and play a role in stimulating melanogenesis [20]. Therefore, inhibiting phosphorylation activation of CREB, AKT, or GSK may result in hypopigmented melanocytes [21]. p-CREB expression was significantly reduced (26 %, Fig. 2a) in cells stimulated with *p*-coumaric acid (1 mM), while p-AKT and p-GSK expression were not affected. These results suggest that the inhibitory effects of *p*-coumaric acid on the MITF-dependent melanogenic mechanism were induced mainly by suppressing p-CREB during cAMP signaling.

p-Coumaric acid reduces the expression of MITF and tyrosinase

Next, we investigated the expression of genes and proteins in melanogenesis including MITF, a master regulator of melanogenesis and its target gene, and tyrosinase. *p*-Coumaric (1 mM) acid reduced MITF and tyrosinase mRNA expression by 73 and 82 %, respectively, compared to the controls (Fig. 2b). The protein expression pattern was similar to that shown for mRNA by qPCR. MITF and tyrosinase protein levels decreased by 27 and 20 %, respectively (Fig. 2c). Protein reductions were more predominant than mRNA down regulations. We believe the protein reduction is primarily due to the transcriptional downregulation but cannot eliminate the involvement of additional mechanisms such as increased ubiquitination or reduced mRNA stability. These together with reduced CREB phosphorylation suggest that *p*-coumaric acid may show hypopigmentation by PKA-dependent reduction in CREB phosphorylation and subsequent downregulation of MITF and tyrosinase expressions in melanocytes.

Although the skin-whitening effects of rice bran are well known, the mechanisms through which the compounds responsible for anti-melanogenesis exert their effects are largely unknown. In this study, we found that *p*-coumaric acid in rice plays a major role in the hypopigmentation effect. *p*-Coumaric acid directly inhibited tyrosinase

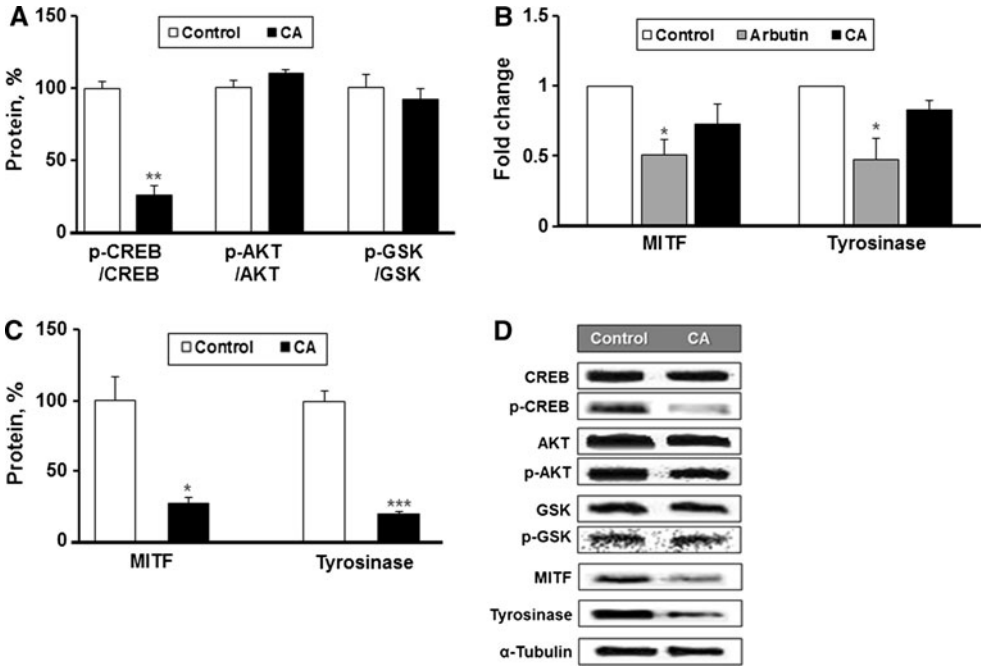

Fig. 2 Effect of *p*-coumaric acid on melanogenesis gene expression. **a** Phosphorylation of PKA target signaling proteins. **b** MITF and tyrosinase expression assessed by qPCR. **c** MITF and tyrosinase protein expression measured by immunoblotting. **d** immunoblot images. $*P < 0.05$ compared to the control (no treatment). Data represent the mean ± SE of three independent experiments

enzyme activity and also reduced melanogenic gene expression by inhibiting CREB phosphorylation. This study provides an understanding of the benefits of natural hypopigmentation sources and will promote the development of novel hypopigmentation products.

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