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ORIGINAL RESEARCH PAPER

Momilactione B inhibits protein kinase A signaling and reduces tyrosinase-related proteins 1 and 2 expression in melanocytes

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Abstract Momilactone B (MB) is a terpenoid phytoalexin present in rice bran that exhibits several biological activities. MB reduced the melanin content in B16 melanocytes melanin content and inhibited tyrosinase activities. Using transcriptome analysis, the genes involved in protein kinase A (PKA) signaling were found to be markedly altered. B16 cells stimulated with MB had decreased concentrations of cAMP protein kinase A activity, and cAMP-response element-binding protein which is a key transcription factor for microphthalmia-associated transcription factor (MITF) expression. Accordingly, the expression of MITF and its target genes, which are essential for melanogenesis, were reduced. MB thus exhibits anti-melanogenic effects by repressing tyrosinase enzyme activity and inhibiting the PKA signaling pathway which, in turn, decreases melanogenic gene expression.

Keywords Momilactone B · Melanogenesis · Protein kinase A · Tyrosinase

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Introduction

Cellular melanin content determines skin, hair, and eye colors. Disorders of melanogenesis cause medical problems, including Addison's disease (Iwata et al. 1990), as well as cosmetic issues. Therefore, the appropriate control of melanogenesis is important for skin health. Melanogenesis is a complicated biological process that is regulated by multiple mechanisms including the synthesis of intermediate chemicals by both transcriptional and post-transcriptional mechanisms (Steingrimsson et al. 2004). Melanin synthesis is initiated from tyrosine in the melanosome of melanocytes (Lerner and Fitzpatrick 1950), and three enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1), and TRP2 (dopachrome tautomerase), play key roles (Costin and Hearing 2007).

Tyrosinase mediates the first two steps of melanin synthesis: the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and its subsequent

oxidation to dopaquinone. TRP2 then catalyzes the conversion of dopachrome to 5,6-dihydrozyindole-2-carboxylic acid (DHICA). TRP1 subsequently oxidizes DHICA to indole-5,6-quinone-2-carboxylic acid, which results in the synthesis of eumelanin and pheomelanin (del Marmol and Beermann 1996). Transcriptional regulation of melanogenic genes is largely mediated by microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix-zip transcription factor (Kim et al. 2010). Thus, chemicals that inhibit MITF expression or function could induce hypo-pigmentation. The expression of MITF is induced by cAMP response element-binding (CREB) protein, which is activated by protein kinase A (PKA) signaling (Levy et al. 2006). The active MITF then upregulates its target melanogenic genes, such a TRP1 and TRP2, and induces melanin synthesis in melanocytes.

Rice bran has been used as a skin-whitening agent for hundreds of years in many East Asian nations, including Korea and Japan (Chung et al. 2009). To identify anti-melanogenic rice chemicals, we previously isolated 18 compounds from rice and performed anti-melanogenic activity screening; momilactione B (MB) was identified as a key anti-melanogenic chemical. MB is a terpenoid phytoalexin found in rice bran (0.5 μg/g dry rice husk) that has several biological activities, including cellular growth inhibition, allelopathy, and antitumor activity (Kato et al. 1973; Kim et al. 2007). Here we report the effect of MB on melanogenesis in B16 murine melanocytes.

Materials and methods

Materials

B16 murine melanocytes were purchased from the Korean Cell Line Bank (Seoul, Korea). Purified momilactione B (MB) was provided by the National Institute of Crop Science in Korea. Arbutin, mushroom tyrosinase, protease inhibitor cocktail, iQ SYBR Green Supermix and BSA from Sigma. TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA), while PowerScript Reverse Transcriptase was obtained from Clontech (Mountain View, CA, USA). Protein nitrocellulose transfer membranes were purchased from Whatman (Dassel, Germany). The ECL western blotting analysis system was from Amersham Biosciences (Piscataway, NJ, USA). Protein quantification reagent was obtained from Bio-Rad and the Fontana-Masson staining kit was purchased from American MasterTech Scientific, Inc. (Lodi, CA, USA). The cAMP enzyme immunometric assay and PKA kinase activity kits were purchased from Enzo Life Sciences (Farmingdale, NY, USA). For the microarray work, RNeasy mini, RNase-Free DNase, and PCR purification kits were purchased from Qiagen (Hilden, Germany). Cys3-UTP and Cys5-dUTP were obtained from Amersham Biosciences (Piscataway, NJ, USA). The Mouse OneArray chip was produced by Phalanx biotech group (Hsinchu, Taiwan).

Cell culture

B16 murine melanocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml, and 0.1 mg streptomycin/ml at 37°C in a humidified atmosphere of 95% air and 5% CO_2, as described previously (Lee et al. 2010).

Mushroom tyrosinase assay

Dopaoxidase activity of mushroom tyrosinase was determined based on a method described previously, with minor modifications (Lee et al. 2011b). Tyrosine (50 μl, 0.03% in distilled water), 75 μl 0.1 M phosphate buffered saline (PBS; pH 6.8) with or without the test sample were added to a 96-well microplate, and then 25 μl mushroom tyrosinase (400 U/ml in 0.1 M phosphate buffer) was added. After incubation at 37°C for 20 min, the amount of dopachrome produced in the reaction mixture was determined at 475 nm. Inhibitory effects on enzyme activity by either MB or arbutin (positive control) are presented as % of inhibition, [1-(sample A_{475}/control A_{475})] × 100 as compared with PBS (negative control) (Choi et al. 2010).

Cellular tyrosinase assay

Tyrosinase activity in B16 cells was assayed as described previously with minor modifications (Lee et al. 2011b). Cells were washed twice with ice-cold PBS and lysed with 20 mM Tris/HCl + 0.1% Triton X-100 (pH 7.5). Tyrosinase activity was analyzed by quantifying the oxidation of DOPA to dopachrome at

475 nm after 2 h. The reaction mixture, containing 140 µl freshly prepared substrate solution [0.1% L-DOPA in 0.1 M sodium phosphate (pH 6.0)] and 70 µl enzyme solution, was incubated at 37°C. Auto-oxidation of L-DOPA was adjusted for normalization. Enzyme activities were expressed as a percentage of the control groups.

MTT assay

The viability of cultured cells was determined based by the amount of MTT reduced to formazan (Chung et al. 2008). After treatment with MB or arbutin, culture medium containing MTT (0.5 mg/ml) was added to each well, and the cells were incubated at 37°C for 3 h before mixing with DMSO to dissolve the formazan crystals. The absorbance at 570 nm was then measured. Values were normalized to the samples' protein concentration.

Cellular melanin content

Cells were washed with PBS and lysed with 20 mM Tris/HCl + 0.1% Triton X-100 (pH 7.5). After centrifugation, pellets were dissolved in 1 M NaOH for 1 h at 60°C. The melanin content of the samples was measured at 400 nm. Cellular melanin content was adjusted according to the samples' protein concentration. The protein concentration was determined using a Bradford protein kit.

Fontana-Masson staining

B16F1 melanoma cells that were cultured on slides were fixed with 4% (v/v) paraformaldehyde for 30 min at room temperature and stained for melanin using a Fontana-Masson staining kit according to the manufacturer's instructions. Briefly, cells were stained with ammoniacal silver solution of 60°C and then treated with 0.1% $AuCl_3$ for 1 min, and 5% (w/v) $Na_2S_20_3$ for 5 min.

Real-time PCR

Total RNA was extracted from cells using a TRI Reagent kit according to a method reported previously (Lee et al. 2011a) and suspended in diethylpyrocarbonate (DEPC)-treated water. For cDNA synthesis, 2 µg total RNA was reverse transcribed with PowerScript Reverse Transcriptase according to the manufacturer's instructions using a combination of oligo-dT primer and random hexamers. Real-time reverse transcriptase (RT)-PCR was then performedin 25 µl using 12.5 µl iQ SYBR Green Supermix, 0.5 µl of each primer (15 µM), 1 µl of cDNA, and 10.5 µl sterile water. Real-time PCR was performed using: one cycle of 3 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C with final extension for 5 min at 72°C. Following amplification, DNA melting curves at temperatures between 54 and 95°C with a heating rate of 0.2°C per second were generated. All real-time PCRs were performed using an iCycler iQ (Bio-Rad, USA). During the primer extension step, the increase in fluorescence from DNA amplification was recorded using the SYBR Green optic channel at a wavelength of 490 nm. Data were collected and viewed using the iCycler iQ optical system software ver. 3.1 (Bio-Rad, USA).

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris/HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, pH 7.5) containing protease inhibitor cocktail at 4°C. Protein concentration was determined using Bradford reagent with BSA as a standard. Protein samples in Laemmli sample buffer were boiled for 5 min and separated on 7.5% SDS-PAGE) gels. Separated proteins were electrophoretically transferred onto nitrocellulose membranes. Non-specific binding was blocked with 5% (v/v) non-fat dry milk in Tris buffered saline/Tween 20 (TBS-T) buffer for 1 h at RT, and membranes were incubated with primary antibodies overnight at 4°C. The levels of p-CREB, MITF, TRP1, TRP2, and tyrosinase proteins were analyzed using specific antibodies diluted (1:500) in 5% skim milk. After washing several times with 1% TBS-T wash buffer, membranes were incubated with the secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence western blotting detection reagents and quantified with the ChemiDoc XRS system (Bio-Rad, USA) using the Quantity One software (ver.4.6.9, Bio-Rad) (Park et al. 2011).

Direct cAMP measurement

Cells were lysed with 0.1 M HCl for 10 min at RT and then centrifuged at 600×*g*. cAMP levels were quantified using a cAMP enzyme immunometric assay kit according to the manufacturer's instructions. The measured cAMP levels were normalized to samples' protein concentrations.

Protein kinase A activity assay

PKA activity was determined using an ELISA kit according to the manufacturer's instructions. The cells were harvested after treatment for 72 h and total protein concentrations were measured in order to normalize the results. Active PKA was used as a positive control.

Microarray analysis

B16 cells were treated with MB (3 μM) for 72 h, and then subjected to microarray analysis after RNA isolation. To remove any contaminating DNA, on-column digestion with RNase-free DNase was performed and samples were then cleaned-up using an RNeasy elutes kit. Purified total RNA (8 μg) was used for cDNA synthesis; the cDNA was subsequently labeled with Cy3-dUTP or Cy5-dUTP. Labeled cDNA samples were purified once and hybridized with arrays for 15 h. The arrays were then scanned, and the data visualized using GenePix ver. 4.0 software. Candidate melanogenesis-related genes were selected, and fold-changes in expression were calculated.

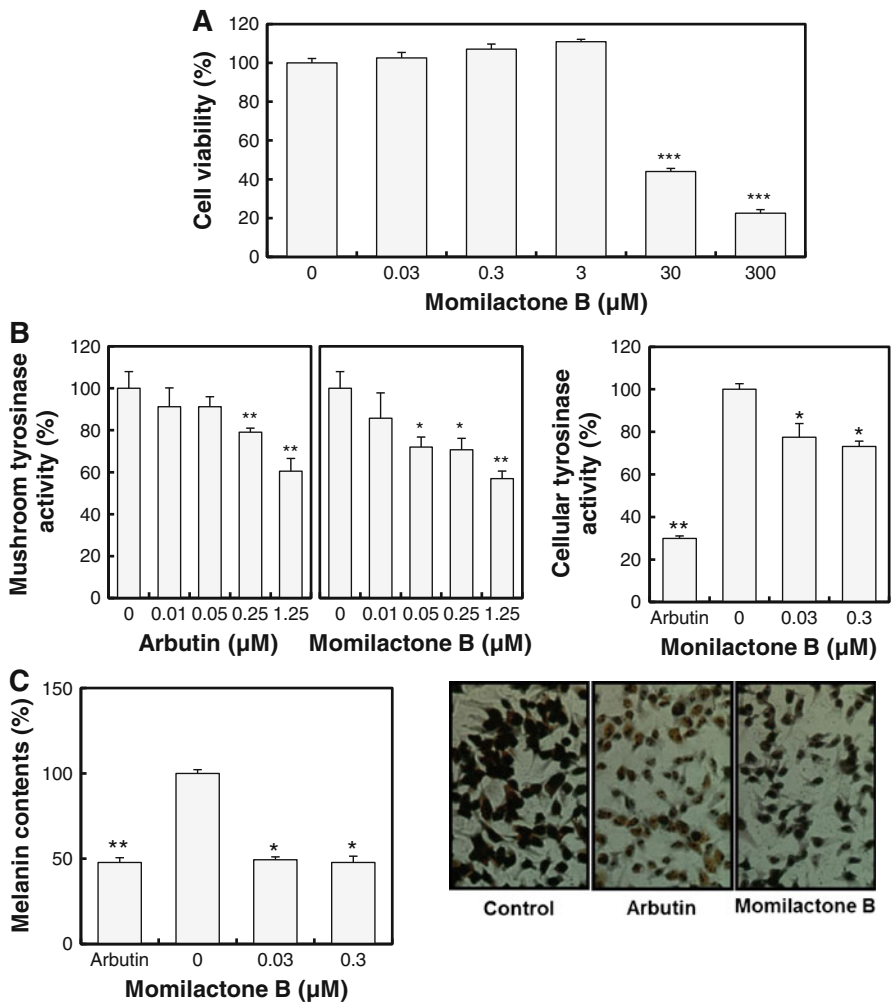

Fig. 1 Effects of momilactone B on melanogenesis. **a** B16 melanocyte viability after stimulation with MB, assessed using an MTT assay. **b** Mushroom and cellular tyrosinase activities. **c** Melanin content of B16 cells. Melanin within B16 murine melanocytes was visualized by Fontana-Masson staining, as described in the "Materials and methods" section. Melanin is stained *dark-brown*. *Values* in *bar graphs* were normalized to sample protein concentrations. The data are the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.005$

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Student's *t*-tests were performed to compare data between groups. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Rice bran has anti-melanogenic activity and therefore is widely used as a depigmentation compound in East Asian nations. However, the key depigmenting compounds in rice bran remain largely unknown. We initially isolated 18 rice compounds and investigated their hypopigmentation effects in vitro (data is not shown). Here, we report that momilactone B (MB) exhibits a significant hypo-pigmentation effect. It is a terpenoid phytoalexin isolated from rice bran that possesses several biological activities. MB exerts potent cytotoxic effects on a human colon adenocarcinoma cell line, thus HT-29 and SW620 cells treated with MB (0.5 μM) showed approx. 20% reduced cell viability (Kim et al. 2007); however, MB did not affect the viability of B16 murine melanocytes up to 3 μM in an MTT assay (Fig. 1a). Thus we examined the anti-melanogenic activity of MB at non-toxic concentrations (<3 μM).

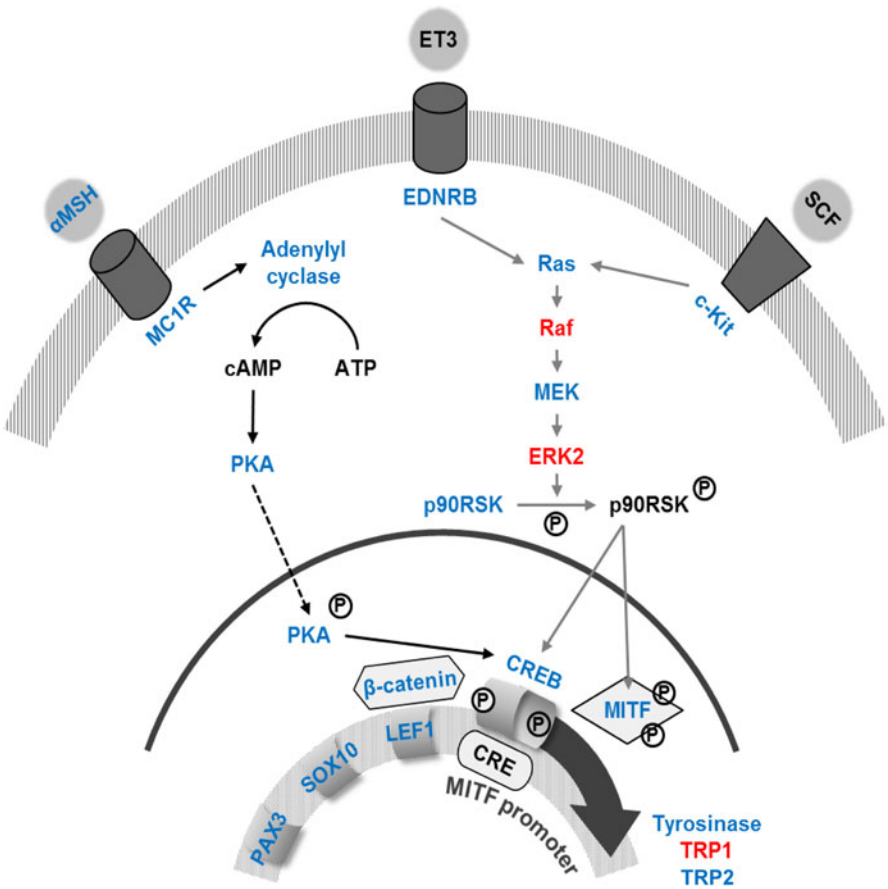

Fig. 2 Expression of PKA and ERK signaling pathways and melanogenesis-related genes. Transcript levels were determined by microarray analysis. Genes whose expression was downregulated are shown in *blue*, while *red* indicates upregulation. *αMSH* alpha-melanocyte stimulating hormone, *MC1R* melanocortin-1 receptor, *PKA* protein kinase A, *PAX3* paired box 3, *SOX10* sex determining region Y-box 10, *LEF1* lymphoid enhancer-binding factor 1, *TCF* transcription factor, *ET1* endothelin 1, *EDNRB* endothelin receptor type B, *c-Kit* proto-oncogene tyrosine-protein kinase kit, *Ras* GTPase H Ras, *Raf* RAF proto-oncogene serine/threonine-protein kinase, *MEK* mitogen-activated protein kinase kinase 1, *ERK* extracellular signal-regulated kinase, *p90RSK* p90 ribosomal S6 kinase, *CREB* cAMP response element-binding protein, *CBP* CREB-binding protein, *MITF* microphthalmia-associated transcription factor, *TRP* tyrosinase-related protein

MB had a direct inhibitory effect on mushroom tyrosinase activity in a dose-dependent manner (Fig. 1b); surprisingly, this effect was stronger than that observed for arbutin. The inhibitory effect of MB on cellular tyrosinase activity and melanin content was further confirmed using B16 murine melanocytes. Stimulation of melanocytes with MB significantly reduced both cellular tyrosinase activity and melanin content. Cellular tyrosinase activity was significantly decreased by 23 and 27% ($P = 0.03$, 0.002) using 0.03 and 0.3 μM MB, respectively, as compared with non-treated controls (Fig. 1b). Cellular melanin content was decreased by 51 and 52% by 0.03 and 0.3 μM MB, respectively, ($P = 0.03$ for both; Fig. 1c). Compared with mushroom tyrosinase activity, cellular tyrosinase activity and melanin content did not respond dose-dependently to MB and low concentrations showed significant effects. The effect of MB on cellular melanin content was comparable to that of arbutin (42% reduction; $P = 0.0001$). Fontana-Masson staining showed similar results. Cellular distribution of melanin pigment was markedly decreased after MB stimulation (Fig. 1c). These data suggest that MB may exert its anti-melanogenic effect by direct inhibition of tyrosinase enzyme activity, the rate-limiting enzyme in melanogenesis.

Melanogenesis is a complex process regulated by multiple mechanisms. The PKA and extracellular signal-related kinase (ERK) signaling pathways are critical, non-genomic regulatory mechanisms (Fig. 2). We performed transcriptome profiling to analyze the expression of genes in the PKA and ERK pathways, as well as key genes in melanogenesis that might be involved in the anti-melanogenic activity of MB (Table 1). Interestingly, stimulation of B16 cells with MB altered the expression of PKA pathway genes. The expression of α-melanocyte stimulating hormone (α-MSH), MC1R, and PKA was changed by −23, −33, and −22%, respectively, while genes in the ERK pathway were only marginally affected in MB-stimulated cells (Fig. 2). MB stimulation suppressed the expression of the transcription factors lymphoid enhancer-binding factor-1 (LEF1), SRY (sex determining region Y)-box 10 (SOX10), and paired box 3 (PAX3), as well as CREB (−40%). LEF and β-catenin, which can synergistically affect the expression of the 1 M promoter, were downregulated (−42 and −36%, respectively). Furthermore, reduced expression of MITF (−20%) and melanogenic genes, including TRP2, was detected by microarray analysis.

Table 1 The expression of genes in melanogenesis altered by MB stimulation in B16 cells

Gene	Fold change
Genes in PKA signaling pathway	
αMSH	0.73
MC1R	0.67
Adenylyl cyclase	0.91
PKA	0.78
Genes in ERK pathway	
ET1	1
EDNRB	0.78
c-Kit	0.9
Ras	0.9
Raf	1.11
MEK	0.87
ERK	1.15
p90RSK	0.66
Genes in nucleus	
PAX3	0.87
SOX10	0.87
LEF1	0.58
TCF	0.62
β-Catenin	0.64
CREB	0.6
CBP	0.74
MITF	0.8
Melanogenic enzymes	
Tyrosinase	0.77
TRP1	0.98
TRP2	0.63

Results of the gene expressions are summarized in Fig. 2

The effect of MB on PKA signaling and the expression of genes related to melanogenesis is shown in Fig. 3. Stimulation of cells with MB significantly reduced cytosolic cAMP concentrations (Fig. 3a; −21% at 0.3 μM; $P < 0.05$) and PKA activity (Fig. 3a; −53% at 0.3 μM; $P < 0.005$). Phospho-CREB (p-CREB) levels were altered by −54% after MB stimulation of B16 cells (Fig. 3b). MB also significantly reduced MITF gene and protein expression by −82 and −56%, respectively (Fig. 3b). Finally, melanogenic gene expression was reduced after MB stimulation. The activities of tysosinase, TRP1, and TRP2 mRNA were changed by −13, −69, and −37%, respectively. Immunoblot data showed similar trends. Tyrosinase, TRP1, and TRP2 protein levels were

changed by −25, −74, and −79%, respectively, as compared with controls. Arburin significantly inhibited tyrosinase enzyme activities; however it did alter the expression of genes and proteins in melanogenesis. This is in line with previously published data that anti-melanogenic activity of arburin is mainly due to tyrosinase inhibition with marginal effects on the expression of genes and proteins in melanogenesis (Chakraborty et al. 1998; Maeda and Fukuda 1996). The results suggest that the alteration of melanogenic gene and protein expression by PKA signaling could lead to reduced melanin synthesis (Fig. 1c). We supposed these results in advance through microarray analysis. Most genes showed correlation between microarray and PCR analysis. TRP1 expression was marginally altered in microarray analysis, however, its expression was down-regulated in qPCR, which is a much more sensitive method, and by immunoblot analysis. Microarray results, indeed, often show significant variability which, detects the intensity of the spot of the array chip while the PCR value is determined by C_t value assessed with fluorescence-labeled amplified cDNA, which is normalized by one reference gene, such as cyclophilin. Thus the results from qPCR are more reliable (Beckman et al. 2004; Morey et al. 2006).

Cellular cAMP activates PKA to upregulate CREB phosphorylation. Active CREB then binds to the M-promoter of MITF, which is a key transcription factor in melanogenesis, as well as other biological pathways, including cell differentiation and proliferation (Huber et al. 2003). Thus, diminished CREB phosphorylation could suppress melanin synthesis via MITF reduction. Anti-melanogenic mechanism of MB differs from that of arbutin, although both MB and albutin similarly reduce cellular melanin content. Arbutin strongly inhibits tyrosinase activity but its effects on gene expression and PKA signaling were

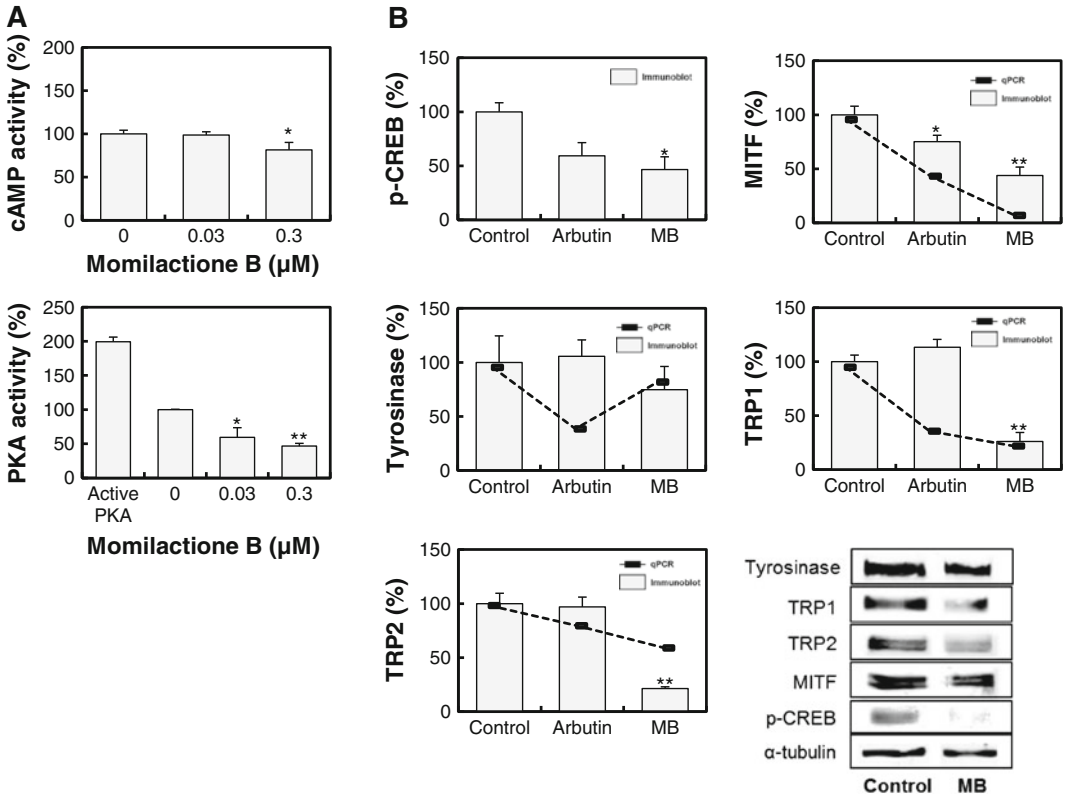

Fig. 3 Effects of momilactone B on the regulation of melanogenic enzymes and transcription factors. **a** Relative cellular cAMP concentrations and protein kinase A (PKA) activity. **b** Immunoblotting and qPCR analysis of phospho-cAMP response element-binding protein (pCREB), microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2. The data are the mean ± SEM, $*P < 0.05$, $**P < 0.005$

marginal. Stimulation of cells with MB decreased TRP1 and TRP2 expression and this could significantly reduce the total melanin content of melanocytes (Fig. 3).

In conclusion, MB decreased cellular melanin content by two mechanisms: (1) direct inhibition and (2) reduced expression of melanogenic genes by the inhibition of PKA and subsequent MITF expression.

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