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Methyl-2-acetylamino-3-(4-hydroxyl-3,5-dimethoxybenzoylthio) propanoate suppresses melanogenesis through ERK signaling pathway mediated MITF proteasomal degradation

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ABSTRACT

Background: Microphthalmia-associated transcription factor (MITF) is regulated by expression and/or degradation pathway, controlling to the expression of melanogenic enzymes for melanin synthesis. Methyl-2-acetylamino-3-(4-hydroxyl-3,5-dimethoxybenzoylthio)propanoate (MAHDP) is reported to anti-melanogenesis effect but its mechanism remain unclear.
Objective: To investigate the effects of MAHDP on melanogenesis and elucidate its mechanism.
Methods: Tyrosinase activity, melanogenic proteins and gene expression levels were measured with MAHDP treatment in B16F1 cells, human melanocytes, reconstructed skin and clinical trial.
Results: MAHDP attenuated melanin production in α-MSH (melanocyte stimulating hormone) stimulated-B16F1 cells. MAHDP decreased the expression of tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2). But, MADPH did not affect the phosphorylation of p38 MAPK, JNK and AKT, which are associated with the regulation of MITF expression. These results suggest that MITF downstream is regulated not transcriptionally but translationally. Treatment of MG132 (a proteasomal degradation inhibitor) almost abolished the decrease of MITF protein levels by MAHDP. Phosphorylation and ubiquitination of MITF for proteasomal degradation were increased by treatment of MAHDP. Treatment of PD98059 (an ERK phosphorylation inhibitor) abrogated ERK phosphorylation, downregulation of MITF and tyrosinase as well as the decrease of melanin contents by MAHDP. Therefore, the degradation of MITF proteins by MAHDP is regulated to the ERK signaling. Finally, MAHDP improved the pigmentation in human epidermal melanocytes, a UVB-irradiated the reconstructed skin model and clinical trial without cytotoxicity and skin irritation.
Conclusion: These results clearly demonstrate that MAHDP suppresses the expression of melanogenic enzymes through ERK phosphorylation-mediated MITF proteasomal degradation, and suggest that MAHDP may be efficient as a therapeutic agent for hyperpigmentation.

1. Introduction

Melanin is synthesized for protecting the skin against extensive environmental stimuli a deficiency of epidermal melanin induces the increase of skin aging as wrinkle and hyperpigmentation such as hypermelanotic skin disorders such as melisma, ephelides, and solar lentigines [1,2]. Melanin production is increased in skin melanocytes following the secretion of the hormones in UV-stimulated keratinocytes [3]. During production of melanins, melanogenic enzymes such as tyrosinase (TYR), tyrosinase related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2) was synthesized [1,4]. Tyrosinase is known for the rate-limiting enzyme and catalyzes oxidation of tyrosine in 2 step; the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone [5]. Dopaquinone converts to dopachrome spontaneously. TRP-2, dopachrome tautomerase, isomerizes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Subsequently, DHICA is polymerized to eu-melanin by TRP-1 [6]. These enzymes up-regulate and activate melanin synthesis.

The microphthalmia-associated transcription factor (MITF), a major transcription factor, has been shown to bind to an M-box motif in the promoter region of melanin genes and up-regulates their transcription. This leads to proliferation and melanogenesis

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of melanocytes that regulate melanogenic enzyme: TYR, TRP-1, and TRP-2 [7]. MITF expression can be regulated various pathway. First, α-Melanocyte stimulating hormone (α-MSH) binds to the melanocortin 1 receptor (MC1R) belong to the G-protein receptor family in melanocytes, which subsequently increases intracellular levels of cAMP, and activates protein kinase A (PKA). This leads to the phosphorylation of cAMP responsive element binding protein (CREB), which can upregulate MITF expression [8]. Suppression of PI3K/AKT pathway is also reported to increase in the activation of GSK3β, which phosphorylates MITF for the upregulation of the expression of melanogenic enzyme [9]. MITF expression can also be controlled by several mitogen-activated protein kinase (MAPK) signaling pathways. Especially, c-Jun N-terminal kinase (JNK) and p38 MAPK has been reported to induce the upregulation of MITF, leading to increase in TYR and TRPs proteins [10].

Melanogenic proteins can be regulated by either proteasomal and/or lysosomal degradation pathways that play a role in the removal of abnormal or misfolded proteins during the maturation process [11,12]. Previously, the proteasomal pathway was reported to remove MITF or TYR, through ubiquitin-mediated degradation endogenously [13]. Especially, the activation of extracellular signal–regulated kinases (ERK) by cAMP-mediated Ras acceleration phosphorylates MITF at serine-73, leading to MITF ubiquitination and subsequent proteasomal degradation, finally inducing the decrease in melanogenic proteins synthesis and melanin biosynthesis [14,15]. As another degradation mechanism, lysosomal enzymes such as glycosidases, proteases, sulfatase etc. can degrade melanogenic enzyme after maturation at the membranes of the Golgi complex. In recent, *Euryale ferox* Seed Extract induce the decrease in TYR protein via lysosomal degradation pathway no inhibitory activity of lysosomal enzyme [16]. Some agents were also reported to increase degradation of tyrosinase [17–19] and MITF through proteasomal/lysosomal degradation pathway [20,21]. Thus, some whitening agents play an important role in their regulation by the degradation of melanogenic proteins and/or MITF transcription factor during melanogenesis for hypopigmentation.

Recently, whitening agents have been synthesized for clinical use against hyperpigmentary disorders and skin whitening effects [1,10]. However, there is a major problem regarding the safety of new compounds because they may have cytotoxicity. The most cytotoxic compounds possess selective cytotoxicity and autoreactivity in melanocytes, and can be also converted by TYR-catalyzed oxidation [22,23]. Specifically, 4-(4-hydroxyphenyl)-2-butanol (rhododendrol) and 4-(4-hydroxyphenyl)-2-butanone (Raspberry ketone) are phenolic compounds that are similar to tyrosine and can be used as an alternative substrate. However, they are converted to unwanted cytotoxic compounds by oxidation via TYR. [24–26]. The compounds possess a p-hydoxyphenyl group, which is a well-known active site for whitening agents (ex. rucinol, hydroquinone and arbutin) [27–30]. For these reasons, it is important to consider the unexpected conversion of whitening agents by oxidation and determine their stability and safety. Recently, we developed novel cysteine derivatives as whitening agents that contain syringic acid and cysteine moieties (L-cysteine ethyl ester, N-acetyl cysteine methyl ester or N-acetyl cysteine ethyl ester), which is biocompatible and safe [31–33]. They possesses the antioxidative and/or whitening effects. Specifically, methyl-2-acetylamino-3-(4-hydroxyl-3,5-dimethoxybenzoylthio) propanoate, MAHDP, indicated a high depigmenting effect without antioxidant and TYR inhibitory effects. However, the mechanism of MAHDP, which is a benzoylsulfanyl acetylcysteine derivative, on melanogenesis are unclear.

In the current study, we investigated the effects of MAHDP on α-MSH-induced melanogenesis in murine melanoma cells (B16F1 cell), and elucidated its mechanism. In particular, we investigated the variation of the ERK signaling pathway and the associated MITF regulation. In addition, depigmenting effects of MAHDP were measured in human epidermal melanocytes (HEM cells), a reconstructed human skin model and clinical trial.

2. Materials and methods

2.1. Chemicals and reagents

Methyl-2-acetylamino-3-(4-hydroxyl-3,5-dimethoxybenzoylthio)propanoate (MAHDP) was synthesized and offered from Daebong LS. Ltd. (Incheon, Korea) [33], Dimethyl sulfoxide (DMSO) mushroom tyrosinase and α-MSH were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

Human epidermal melanocytes isolated from moderately pigmented neonatal foreskin (HEM) were cultured in medium 254 supplemented with human melanocyte growth supplement (HMGS), which were purchased from Cascade Biologics (Portland, OR, USA). B16F1 murine melanoma cells were obtained from Kyung Hee University Skin Biotechnology Center (Seoul, Korea), and were maintained in Dulbecco's modified Eagle's medium (DMEM; Capricorn, Germany) supplemented with 10% fetal bovine serum (FBS; Capricorn, Germany) and 1% penicillin-streptomycin. Cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Measurement of cell viability

Cell viability assay was performed using 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by measuring the reduction value of MTT to formazan. HEM and B16F1 cells were seeded at a density of 1×10^4 cells/well in a 96-well plates. Then, cells were treated indicated concentration of compounds. After incubation for 72 h, the medium was removed and washed, and MTT solution (200 μL) was added to each well. After cells were treated for 1 h, the media is removed and added DMSO for solubilization of formazan. Then, absorbance was measured using a microplate reader (TECAN, Salzburg, Austria) at 570 nm.

2.4. Measurement of melanin content

B16F1 and HEM cells were seeded at 1×10^5 cells/well in 6-well culture plates. Then, cells were treated with indicated concentration of MAHDP for 72 h. B16F1 cells were stimulated with 200 nM α-MSH together. To analyze intracellular melanin content, cells were washed twice with PBS and dissolved in 1 N NaOH solution containing 10% DMSO at 80 °C for 2 h. The cell lysates was transferred to a new 96 well plate and determined by estimating the absorbance at 405 nm using a microplate reader.

2.5. Various TYR inhibitory activity

To estimate TYR inhibitory activities in various species, mushroom tyrosinase was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and cellular TYR was obteined from murine melanocytes (B16F1 cell) and human melanocyte (HEM cell) respectively. B16F1 and HEM cells harvested and lysed using stripper and RIPA buffer (cell lysis buffer). The cell lysates centrifuged at 13,000 rpm for 30 min at 4 °C and supernatant was collected. The protein concentrations in lysates were determined using the BCA protein assay kit (Pierce, Biotechnology, Rockford, IL, USA). Then, the lysate containing the same amount of protein (60 μg) was placed in a 96-well plate and treated with added 10 μL L-DOPA (10 mM) and indicated concentrations of

MAHDP or arbutin for 30 min at 37 °C. The absorbance was measured at 475 nm using a microplate reader.

2.6. Western Blot analysis

After washing with PBS, cells were lysed in RIPA buffer containing protease inhibitors and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was collected and assayed for protein concentration using the BCA kit (Pierce, Rockford, IL, USA). Total protein (60–80 μg) was separated by electrophoresis on 10% SDS-polyacrylamide gels, and transferred to PVDF membranes (Millipore, USA). Blocking was performed in Tris-buffer saline containing 5% skim milk powder (or 5% BSA (Affymetrix Inc., USA)) and 0.1% Tween-20 (Sigma–Aldrich, USA). Samples were incubated the appropriate primary antibodies at a dilution of 1:1000. Primary antibodies against anti-tryosinase, anti-TRP1, anti-TRP-2 and anti-MITF antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA), and anti-phospho-MITF (Serin73) antibody was purchased from Assay Biotechnology Company (Sunnyvale, CA, USA). After washing three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Mouse anti-GAPDH antibody and horseradish peroxidase-conjugated secondary antibodies were purchased from Thermo Scientific (USA).Bound antibodies were detected using an enhanced chemiluminescence plus kit (GE Healthcare, USA). The specific bands were analyzed using Image J software.

2.7. Analysis of MITF ubiquitination

To investigate whether or not the MITF protein is ubiquitinated by MAHDP treatment, B16F1 cells were pre-treated with or without MG132 (25 μM) for 1 h, and then treated α-MSH (200 nM) for 1 h. The cells were treated with MAHDP for 3 h. The cells were harvested and lysed with RIPA buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium sarcosyl, 1 mM DTT] plus protease inhibitors. The lysate was precleared with 0.25 μg mouse IgG and 40 μL of protein A/G-agarose (Santa Cruz Biotechnology Inc.) under continuous mixing for 2 h at 4 °C. After centrifugation (3000 rpm for 30 s at 4 °C), the supernatants were used as precleared cell lysates. The precleared cell lysates were incubated with 2 μg of the anti-MITF antibody (Santa Cruz Biotechnology, sc-56725). After overnight continuous mixing at 4 °C, 40 μL of protein A/G-agarose (Santa Cruz Biotechnology, sc-2003) were added for 2 h at 4 °C. The immunocomplexes were precipitated by brief centrifugation, and the pellets were washed three times with 1 mL lysis buffer. Finally, the absorbed proteins were eluted with 2×Tris-glycine SDS sample buffer containing 2-mercaptoethanol at 95 °C for 10 min. Each supernatant was separated on 10% Tris-glycine SDS gels and then transferred to nitrocellulose membranes. Anti-Ub (Santa Cruz Biotechnology, P4D1) was used to detect ubiquitinated MITF precipitated with anti-MITF antibody. The ubiquitinated MITF was analyzed by Western blot analysis.

2.8. Histochemistry of reconstructed 3D skin model

Reconstructed human 3D skin model, Neoderm-ME (Tego Science, Seoul, Korea) consisted of normal human-derived epidermal keratinocytes and melanocytes. In brief, Neoderm-ME was removed from the medium-containing agar and transferred onto 6-well plates for equilibration at 37 °C in 5% CO_2 for 1 day. Under treatment MAHDP or vehicle, Neoderm-ME was irradiated with UVB (20 mJ/cm^2) every other day for a total of fourth exposures, and the tissue samples were kept in an incubator at 37 °C with 5% CO_2. The epidermis was then fixed with 4% formalin in phosphate-buffered saline (PBS) and froze for skin section using cryotome (HM505E, MICROM, Germany) into 10 μm slices followed by Fontana Masson and Hematoxylin and eosin (H&E) method staining to visualize melanin pigments.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolate from cell pellets by RNeasy mini kit (Qiagen, Germany). Total RNA was quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20 μL reaction containing 2 μg of total RNA, oligo (dT), and Reverse Transcription Premix (T&I biotechnology, China). PCR amplification of the cDNA products (3 μL) was performed with PCR premix ELPIS (T&I biotechnology, China) and the following primer pairs (Genotech Daejeon, Korea): MITF forward, 5′-GTA TGA ACA CGC ACT CTC GA-3′, and MITF reverse, 5′-GTA ACG TAT TTG CCA TTT −3′ (135 bp); β-actin forward, 5′-ACT ATT GGC AAC GAG CGG TT-3′, and β-actin reverse, 5′-ATG GAT GCC ACA GGA TTC CA −3′ (81 bp)

2.10. A study of human skin whitening effect of the 1% MAHDP cream

Twenty-one women aged between 20 and 59 years (mean age, 45.3 years) participated in this part of the investigation in a double-blind study. The subjects had the hyperpigmentation around their eyes. The subjects were divided into two groups (group A and group B) and the right and left sides of their faces were allocated to receive either 1% MAHDP cream or vehicle cream, depending on the group to which they had been assigned. 1% MAHDP containing or the vehicle cream was applied twice daily (in the mornings and in the evenings) to their face after washing the face and applying toner. When the study subjects attended the research center for assessments, they rested within the controlled laboratory environment (20–25 °C, humidity 40–60%) for 30 min. The averaged intensities of the designated hyperpigmentation spots were determined after treatment 4 and 8 weeks on the lightness using chroma meter CM700d (Konica Minolta, Japan), and melanin index using mexameter MX18 (Khazaka electronic Gmbh, Germany). This test was performed by P&K Skin Research Center (Seoul, Korea) and conducted according to the human test guidelines regulations based on the Declaration of Helsinki. The change rate of the lightness of the subjects was calculated individually, and then the values were averaged for indicating the change rate of lightness of total subjects. The lightness change rate (%) was calculated as the following equation.

Lightness change rate (%) = (After treatment − Before treatment)/Before treatment × 100

2.11. Statistical analysis

All experiments were carried out in triplicate. Values are presented as mean ± SD Statistical significance of the differences between groups was assessed using analysis of variance (ANOVA).

3. Results

3.1. Effect of MAHDP on melanogenesis in B16F1 melanoma cells

To estimate the inhibitory effects of MAHDP on melanin synthesis in the B16F1 murine melanoma cell line, cells were incubated for 72 h with indicated concentrations of MAHDP, and cell viability was estimated using an MTT assay.

As shown in Fig. 1B, MAHDP indicated no cytotoxicity by 200 μM, and MAHDP (10–200 μM) suppressed melanin production in a dose-dependent manner on α-MSH-stimulated B16F1 cells. MAHDP significantly decreased melanin production at 200 μM similar to that observed for non-treated cells. Interestingly, MAHDP indicated no TYR inhibitory effects in various species (mushroom, murine, or human) (Fig. 1D). Arbutin, which is known

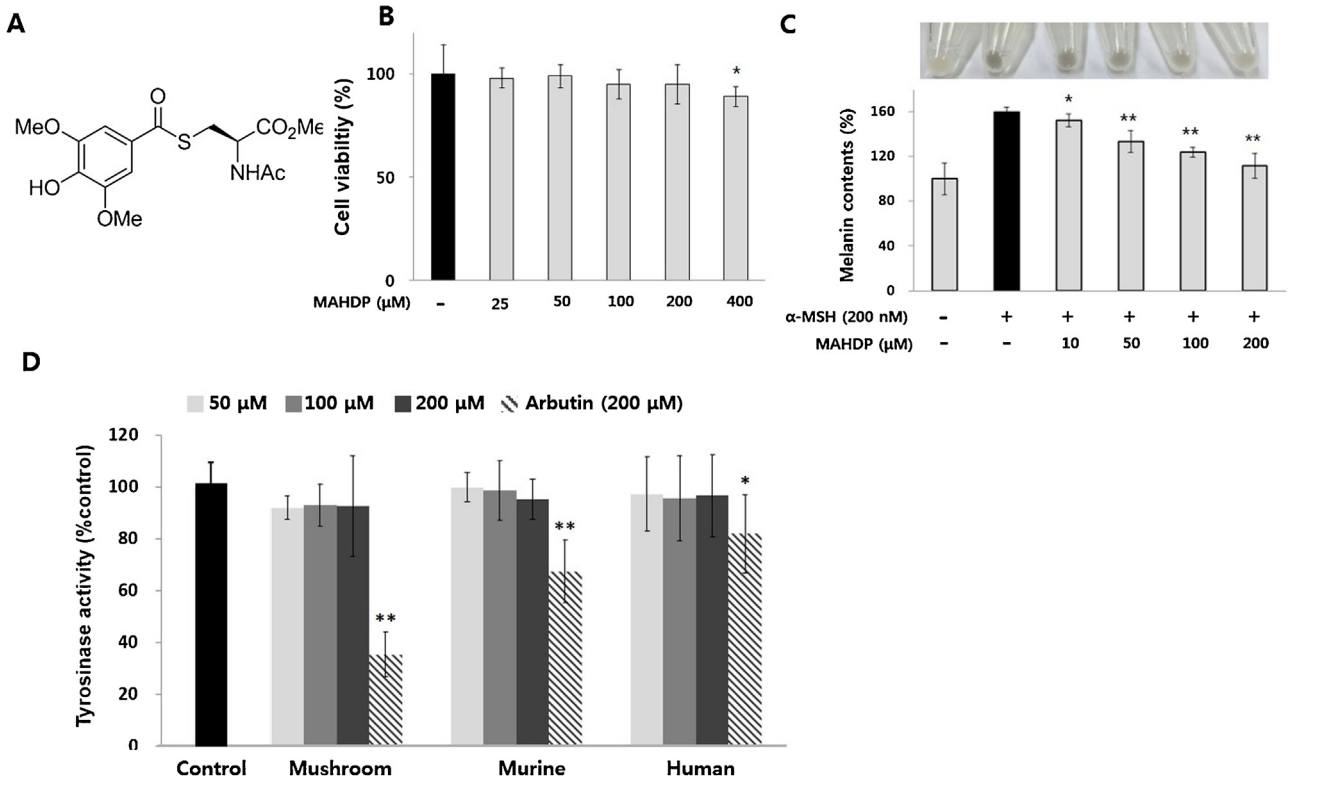

Fig. 1. (A) The chemical structure of MAHDP. (B) Cytotoxicity was evaluated on B16F1 cells treated by MAHDP at the indicated concentrations after incubation for 72 h. (C) B16F1 cells were treated with MAHDP (10, 50,100, and 200 μM) and α-MSH (200 nM). After incubation for 72 h, melanin contents were evaluated at 405 nm. (D) Tyrosinase activity was determined in various hosts by adding 1.0 mM L-DOPA as the substrate, alone or together with MAHDP or arbutin, at the indicated concentrations, and the absorbance of DOPA chrome was read at 490 nm. Each percentage value for the treated cells was reported relative to that of the control cells. Each bar represents the mean ± SD of three independent experiments. Asterisks denote a value that is statistically different (*$P < 0.1$; **$P < 0.01$) in the melanin content compared with non-treated cells (B), α-MSH treatment only (C) or DOPA chrome content without MAHDP treatment as a control (D).

a potent tyrosinase inhibitor, decreased tyrosinase activities in all species [30]. These results suggest that MAHDP possesses a significant depigmentory effect on B16F1 cells, and the whitening mechanism without the inhibitory effect of TYR directly [32].

3.2. MAHDP downregulates melanogenic proteins levels

To elucidate the depigmenting mechanism in α-MSH stimulated B16F1 cells, the effects of MAHDP on the expression of melanin synthesis-related proteins (TYR, TRP-1, TRP-2 and MITF) were analyzed by western blot.

MAHDP markedly decreased TYR expression levels that were similar or lower to those of non-treated cells at all concentration. MAHDP also suppressed protein expressions of TRP-1 from 50 μM MAHDP and decreased TRP-2 expression from 10 μM in a dose dependent manner (Fig. 2A and B). These results suggest that MAHDP suppresses melanogenesis by regulating the expression of melanogenic proteins. Then, we determined the protein levels of MITF after MAHDP treatment in α-MSH stimulated B16F1 cells. MAHDP also decreased MITF levels in a dose-dependent manner (Fig. 2C).

MITF binds melanogenic gene promoters, which increases transcription of melanogenic enzymes, leading to accelerate melanin synthesis [34]. Thus, we investigated the MITF regulatory pathway to determine whether MAHDP contributes to decreased MITF expression levels through transcription or protein degradation. Reverse transcription PCR assays using MITF-specific primers were performed. MITF mRNA level was increased by α-MSH treatment from 10 min on B16F1 cells, and kept for more than 6 h (Supplementary 1A). MAHDP indicated no effects on MITF mRNA levels on B16F1 cells with or without α-MSH (Fig. 2D, Supplementary 1B) as well as the phosphorylation of CRE-binding protein (CREB), which is known for increasing MITF mRNA expression through cAMP-CREB pathway (Fig. 2E). MAHDP also indicated no effects on cAMP level increased by α-MSH treatment (Fig. 2F). These results suggest that the depigmentary effects of MAHDP is involved in mRNA transcription process or protein degradation excluding cAMP-CREB signaling.

3.3. MAHDP decreases MITF expression level through ERK phosphorylation

MITF expression can be regulated by the activation of Akt and MAPK (JNK, ERK and p38 MAPK) [35–37]. Thus, we investigated whether MAHDP regulates the expression of the MITF-associated factors. To elucidate the mechanism by which MAHDP decreases MITF protein expression levels, various MITF downregulation pathways were analyzed by western blot. We pre-treated α-MSH on B16F1 cells for 1 h because the signals is stabilized and kept same protein levels after 1 h of treatment of α-MSH (Supplementary 2A).

As shown in Fig. 3A and B, MAHDP increased phosphorylation of ERK for up to 1 h without the influence on its total form on α-MSH-stimulated B16F1 cells. However, JNK, p38 MAPK, and Akt were not affected by treatment of MAHDP. ERK phosphorylation was also increased when B16F1 cells were treated by MAHDP only (Supplementary 2B.). These results suggest that the observed suppressive mechanism of MAHDP is connected with the upregulation of the ERK pathway, leading to MITF protein degradation and not to a decrease in transcription [14]. To investigate whether ERK phosphorylation contributes to the downregulation of MITF, we treated with α-MSH and MAHDP for 3 h and determined expressions of the proteins.

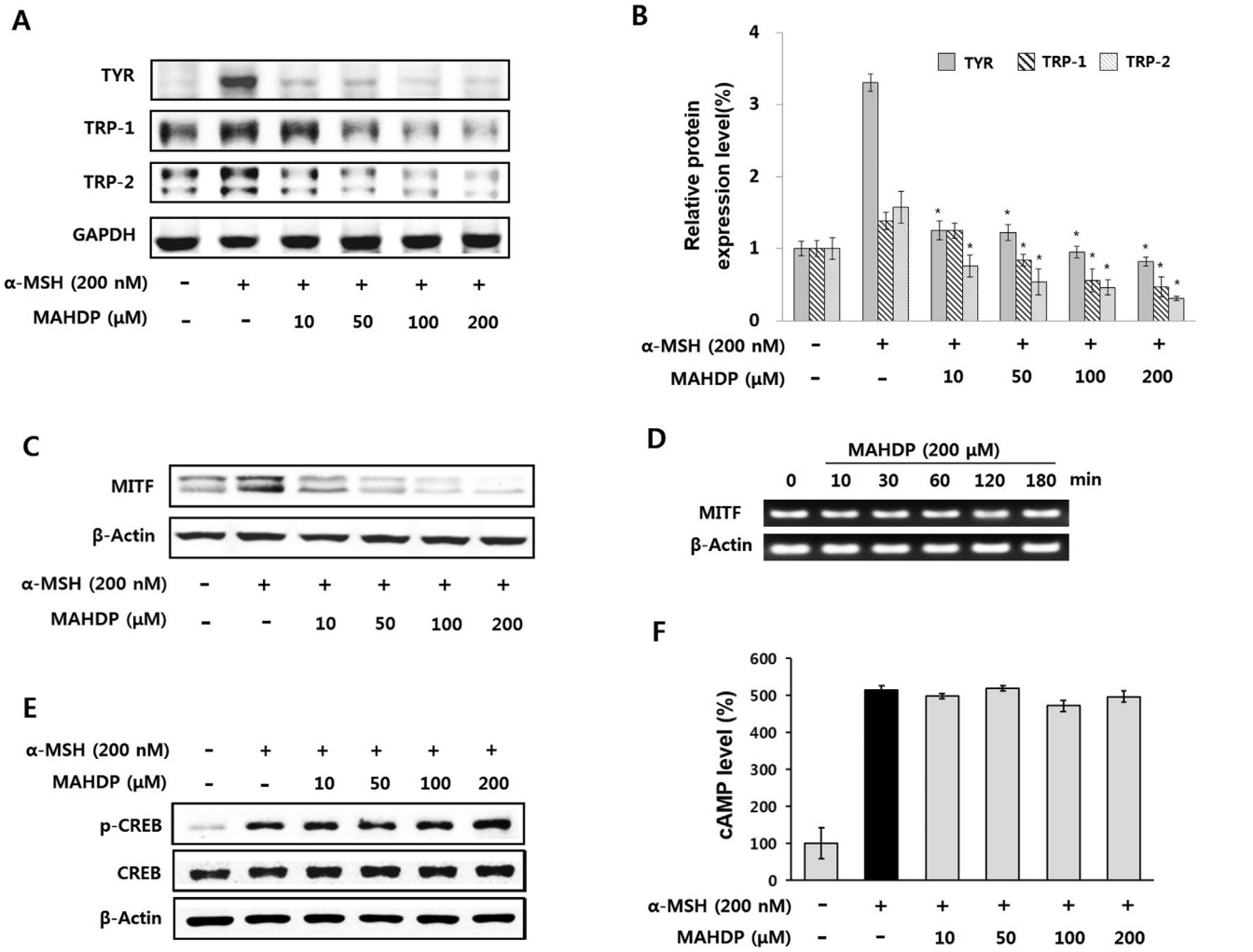

Fig. 2. Tyrosinase (TYR), TRP-1, and TRP-2 were measured in B16F1 cells that were stimulated with 200 nM α-MSH and treated with various concentrations of MAHDP (10, 50, 100, and 200 μM) for 48 h. Protein expressions of TYR, TRP-1 and TRP-2 were analyzed by western blot (A) and was qualified compared to total GAPDH levels using ImageJ program (B). MITF protein was also analyzed by western blot in the same condition (C). (D) MITF mRNA levels were analyzed after 200 μM MAHDP treatment on α-MSH-stimulated B16F1 cells for indicated times. Total RNA was isolated from the cells and used to prepare cDNA. Equivalent amounts of cDNA were amplified with primers specific for MITF, and β-actin primers were used as a control to ensure even loading of target cDNA. The resulting PCR products were analyzed by agarose gel electrophoresis. (E) B16F1 cells were treated with MAHDP in the presence of α-MSH at indicated concentrations for 45 min CREB phosphorylation was analyzed by western blot analysis. (F) B16F1 cells were cultured on 6-well plates and were treated with MAHDP at indicated concentrations under α-MSH for 30 min. cAMP levels were measured using cAMP assay kit (BioVision, USA) at 405 nm. The values represent the mean ± SD of three independent experiments. $^{*}P < 0.05$, compared with the α-MSH treated cells.

As shown in Fig. 3C, treatment of α-MSH increased significantly ERK phosphorylation and decreased MITF protein levels. Phosphor-ERK treated with MAHDP was more increased than with α-MSH alone. To further clarify whether ERK phosphorylation affects to decrease MITF and tyrosinase proteins, we pre-treated with 20 μM PD98059 (an ERK inhibitor) for 1 h before treatment with 200 μM MAHDP. PD98059 treatment relieved the increased ERK phosphorylation and the decreased MITF expression level by MAHDP treatment (Fig. 3C). The decrease of MITF and tyrosinase protein by MAHDP suppressed melanin production on α- MSH-induced B16F1 cells. ERK inhibitor restored the diminished melanin contents by MAHDP (Fig. 3D).

3.4. MITF downregulation by MAHDP was induced by proteasomal degradation pathway

ERK phosphorylation is well known to be closely related with the degradation of MITF protein [15]. Thus, we investigated whether downregulation of MITF expression was caused by proteasomal and/or lysosomal degradation. B16F1 cells were then pre-treated with cycloheximide (a protein synthesis inhibitor) for 1 h, and then pre-treated MG132 (a proteasome inhibitor) or chloroquine (a lysosomal proteolysis inhibitor) for 1 h before being treated with MAHDP for 3 h.

As shown in Fig. 4A, the diminished MITF expression by MAHDP was completely restored by pre-treatment with MG132. However, chloroquine did not affect MITF expression. In a presence of cycloheximide, MAHDP also indicated no effects on tyrosinase protein levels with or without chloroquine or MG132. These results suggest that MAHDP decreased MITF protein specifically through proteasomal degradation.

Proteasomal degradation is caused via the phosphorylation of MITF at Serin 73 by ERK activation, which is followed by MITF ubiquitination and proteasomal degradation [14]. As shown in Fig. 4B, MAHDP increased phosphorylation of MITF at Serin73 and decreased MITF and Tyrosinase protein levels. ERK inhibitor

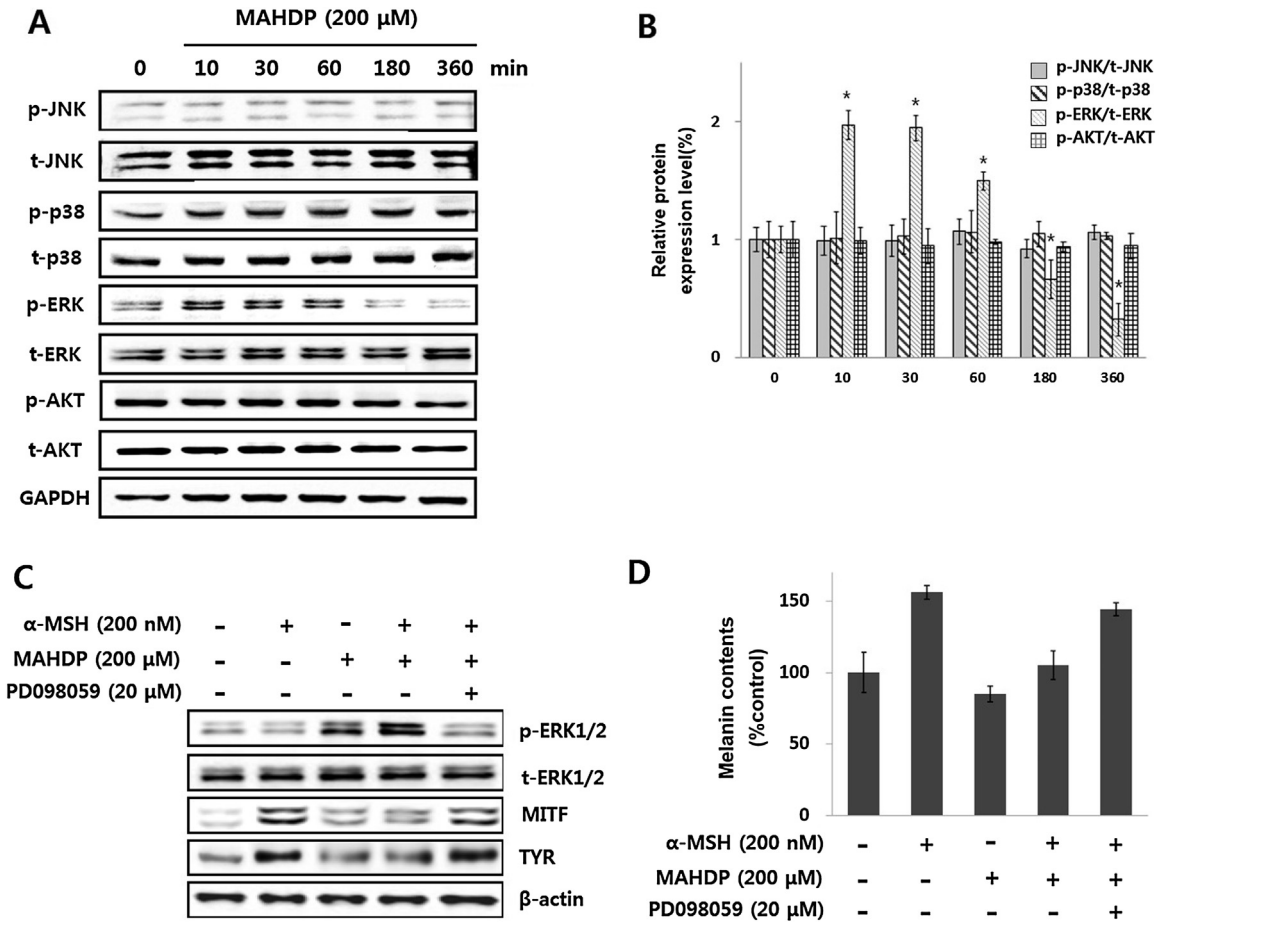

Fig. 3. After serum starvation, B16F1 cells were pre-treated with α-MSH (200 nM) for 1 h, and treated with 200 μM MAHDP in the presence the indicated times. Treated cells were lysed and analyzed via western blot with antibodies against phospho-specific JNK, p38 MAPK, ERK and AKT. (A) Equal protein loading was checked by actin, phosphorylation-independent JNK, p38 MAPK, ERK, and AKT antibodies. (B) Relative intensity of phosphorylated JNK, p38 MAPK, ERK and AKT protein was compared to the control (total form) respectively. B16F1 cells were treated with 200 μM MAHDP in the absence or presence of 20 μM PD98059 and/or α-MSH for 1 h. The cells were harvested and centrifuged for the analysis of the protein levels of phospho-ERK, ERK, MITF and tyrosinase using western blotting (C) and determination of melanin contents (D). The values indicate the mean ± SD for three independent experiments performed in triplicate. *$p < 0.01$ versus protein levels at 0 min.

abolished accelerated MITF phosphorylation and diminished MITF and TYR protein expression. MAHDP also increased MITF ubiquitination (Fig. 4C, lane 4 and 5) compared with non-treated cells by MAHDP (Fig. 4C lane 1–3). Interestingly, MITF ubiquitination is accelerated by MAHDP under α-MSH treatment more than non-stimulated cell by α-MSH. Taken together, these results demonstrate that MAHDP treatment leads to proteasomal degradation of MITF via ERK activation-mediated ubiquitination, which suppresses melanogenic enzyme expression and leads to hypopigmentation.

3.5. MAHDP suppressed melanin production in human epidermal melanocytes and a reconstructed 3D human skin model

We estimated that the suppressive effects of MAHDP on melanin production in human epidermal melanocytes (HEM cells) and a reconstructed human skin tissue model. As shown in Fig. 5A and B, the melanin contents were significantly decreased in a dose-dependent manner after the treatment with MAHDP at 50–200 μM without the apparent changes of cell morphology and cytotoxicity (data not shown). MAHDP increased MITF phosphorylation at serin 73 for 1 h, decreasing MITF proteins completely through proteasomal degradation, and then suppressed tyrosinase expression (Fig. 5C).

The reconstructed human skin was irradiated with ultraviolet B (UVB) in order to induce melanogenesis every other day for 8 days under MAHDP treatment. As shown in Fig. 6, melanin production was increased in UVB-stimulated reconstructed human skin, and MAHDP decreased melanin contents remarkably. MAHDP also suppressed the pigmentation in non-treated reconstructed skin slightly. These results suggest that MAHDP is an anti-melanogenic agent, which is applicable in human skin.

3.6. Whitening effect of MAHDP on hyperpigmented human skin around the eyes

As shown in Figs. 5 and 6, MAHDP suppressed melanin production in HEM cells and reconstructed human skin without cytotoxicity and change of morphology. Thus, we hypothesized that MAHDP can improve a darker human skin. To further determine whether MAHDP is an applicable whitening ingredient for cosmetics, whitening effect of 1% MAHDP containing cream was applied twice daily (in the mornings and in the evenings) for 8 weeks. The skin whitening improvement effect of the subjects was evaluated by the variations of the lightness and melanin index of the hyperpigmented spot around eyes using the devices after 4 and 8 weeks respectively.

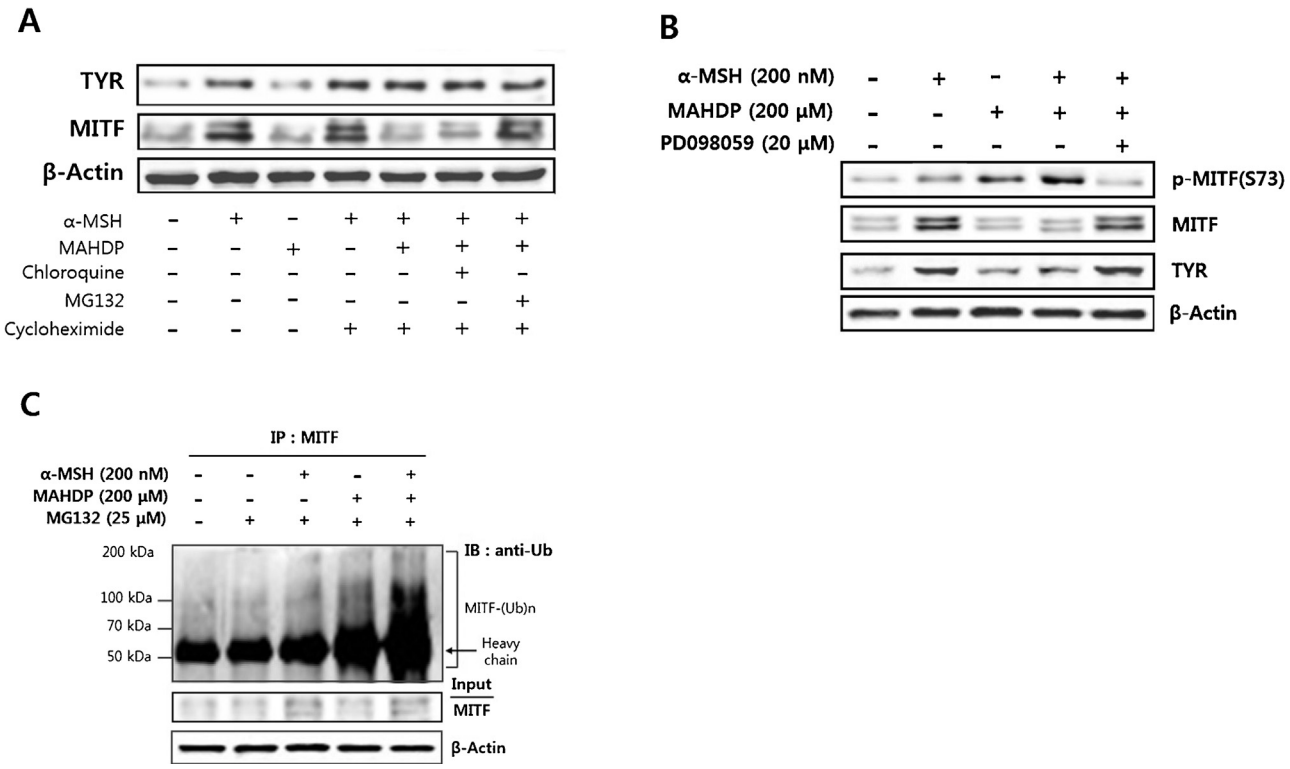

Fig. 4. Effect of MAHDP on MITF proteasomal degradation in α-MSH-stimulated B16F1 cells. The cells were pretreated with or without 25 μg/mL cycloheximide for 1 h, and then pretreated with or without 50 μM. MG132 or 200 μM chloroquine for 1 h, and then treated with or without MAHDP for 3 h. Tyrosinase and MITF proteins expression was analyzed (A). B16F1 cells were pre-treated by ERK inhibitor for 1 h and then treated with MAHDP in a presence or an absence of α-MSH (200 nM). The cell lysates were immunoblotted with the antibody of phospho-MITF (Ser73), total MITF and tyrosinase proteins (B). (C) To investigate MITF ubiquitination, the cell lysates were immnoprecipitated with anti-MITF antibody (IP) and then analyzed ubiquitinated MITF levels using western blotting. Ten percent of the cell lysates were used as the input (Fig. 4C, lower panel). Equal protein loading was confirmed using β-actin.

1% MAHDP containing cream increased the lightness of the hyperpigmented spot around eyes (Fig. 7A), and decreased the M.I of this spot after the treatment 4 and 8 weeks compared with initial time (before) (Fig. 7B) without side effect and skin irritation. These results suggest that MAHDP is considerable to have whitening effect and safety for application as a cosmetic ingredient

4. Discussion

MAHDP, a benzoylsulfanyl acetylcysteine derivative, was consisted with syringic acid and N-acetyl cysteine methyl ester, and possesses weak hydrophobicity [33]. In this study, MAHDP showed remarkable depigmenting effect on B16F1 cells stimulated with α-MSH (Fig. 1C) and HEM cells (Fig. 5A and B) in a dose-dependent manner. MAHDP improved the lightness and decreased the melanin contents on hyperpigmented skin around eyes (Fig. 7A and B) without skin irritation.

MAHDP treatment indicated no inhibitory effects on the TYR activity derived from various types (mushroom, murine, and human) (Fig. 1D) although it decreased melanin production (Fig. 1C). MAHDP is consisted with 3,5-dimethoxy-4-hydroxybenzoic moiety of syringic acid. Especially, a 4-hydroxybenzoic part is known as a potential TYR inhibitor by meta-position of hydroxyl group in benzoic part, which is similar to the structure of tyrosine as the substrate of TYR [38]. However, the steric hindrance of 3,5-dimethoxy groups of MAHDP interrupts that hydroxyl group of MAHDP binds to TYR active site for inhibition of its catalytic activity [38]. In addition, MAHDP was reported to have a low antioxidative activity for the suppression of oxidative reaction in melanogenesis process [33]. Thus, MAHDP suggest that its depigmenting effect was not contributed by TYR inhibitory and antixidative activities but its other mechanisms.

To protect the skin against UV-induced damage, melanin as a protective biopolymer is synthesized in melanosome through the oxidative reaction of melanogenic proteins (TYR, TRP-1 and TRP-2) [39,40]. The expression of these proteins is regulated by MITF, a major transcription factor, containing a helix-loop-helix-leucine zipper domain structure. MITF directly binds to an M-box motif in their promoter site and subsequently increases the transcription of melanogenic proteins. MITF can also regulate melanocyte proliferation, survival and pigmentation [41]. Thus, regulation of MITF expression is important for the melanogenesis in melanocytes [42,43]. In this study, MAHDP significantly suppressed not only TYR, TRP-1 and TRP-2 but MITF protein expressions (Fig. 2A and B). Thus, our data suggest that melanogenesis inhibitory effects of MAHDP are associated with the decrease in MITF protein expression.

MITF expression is regulated mainly via the cAMP-PKA-CREB pathway in α-MSH stimulated melanogenesis [8]. MC1R stimulated by α-MSH activates adenylyl cyclase through G protein signaling, which subsequently accelerates to converse from adenosine-5′-triphosphate (ATP) to cAMP [44]. Then, cAMP activates CREB phosphorylation via a cAMP-PKA-CREB pathway, and subsequently increases MITF mRNA and protein. However, our present results showed that MAHDP treatment did not affect the CREB phosphorylation, expression of MITF mRNA and cAMP acceleration (Fig. 2C-E), but suppressed MITF protein expression (Fig. 2A and B). This suggests that the MAHDP leads to the decrease in MITF protein through its degradation or/and suppression of MITF transcriptional activity.

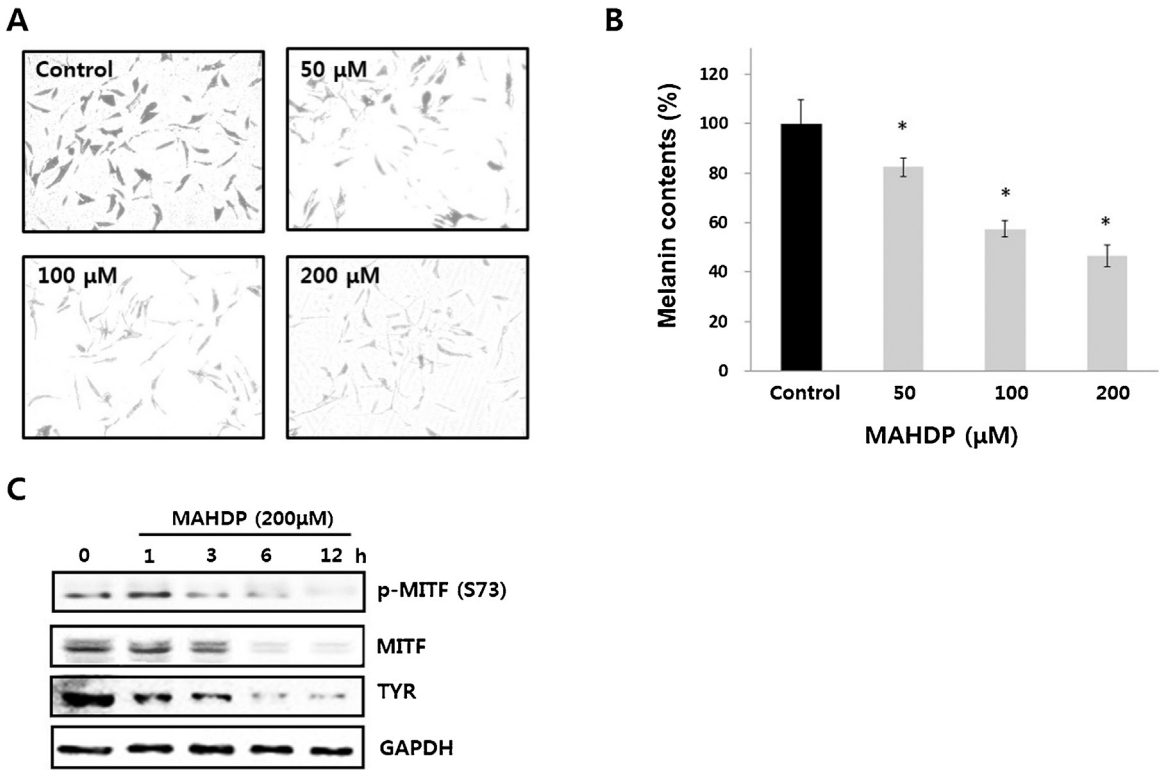

Fig. 5. Effect of MAHDP on melanin synthesis in human epidermal melanocytes (HEMs). HEMs were treated with MAHDP (50, 100, and 200 μM) for 72 h, and stained black using the Fontana–Masson staining method (A), and the cellular melanin contents were measured as described in Section 2.4(B). (C) HEMs were treated by 200 μM MAHDP for indicated times, and then analyzed the expression of phospho-MITF (Serin73), total MITF and tyrosinase (TYR) proteins by western blotting. Representative figures are shown of at least three independent experiments. Data are shown as the mean ± SD. $^{*}P < 0.01$ compared with non-treated cells.

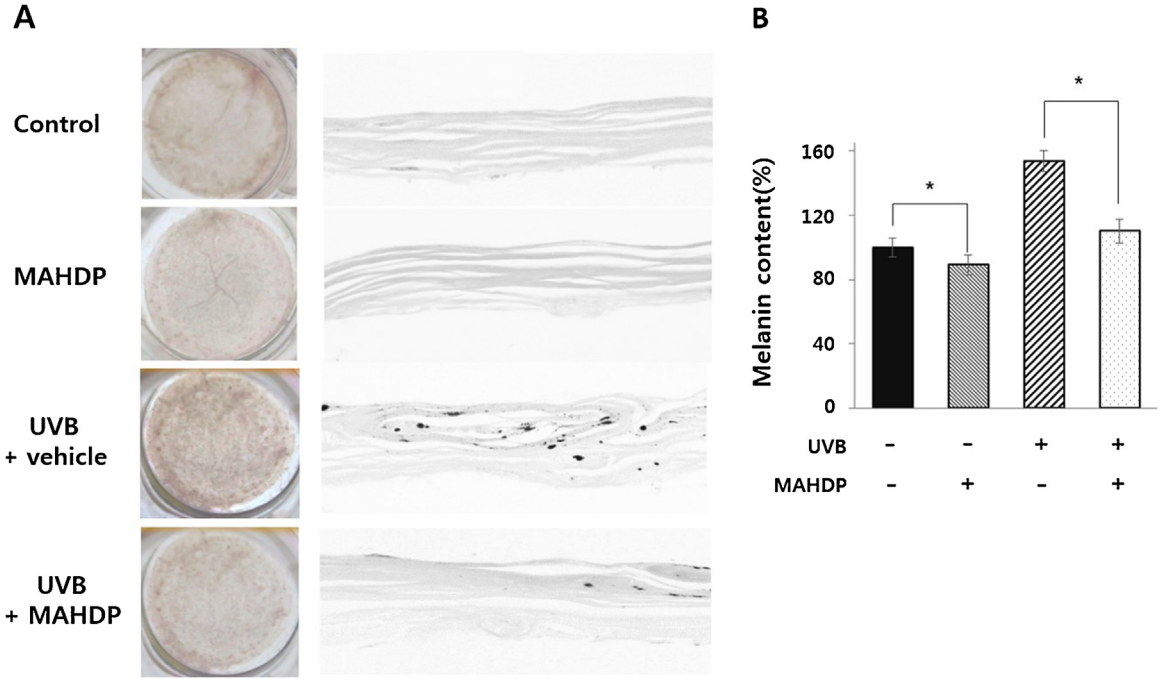

Fig. 6. Inhibitory effect of MAHDP (200 μM) on melanogenesis in a reconstructed human skin model Reconstructed human skin was treated with UVB (20 mJ/cm^2) in the presence of MAHDP every other day for a total of four exposures. (A) Each representative skin image was obtained and melanin pigments were stained with Fontana-Masson and Hematoxylin and Eosin (H&E). (B) The melanin contents of the reconstructed human skin lysates were dissolved in 1 N NaOH, and its supernatants were measured at 450 nm. Data are shown as mean ± SD (n = 3).

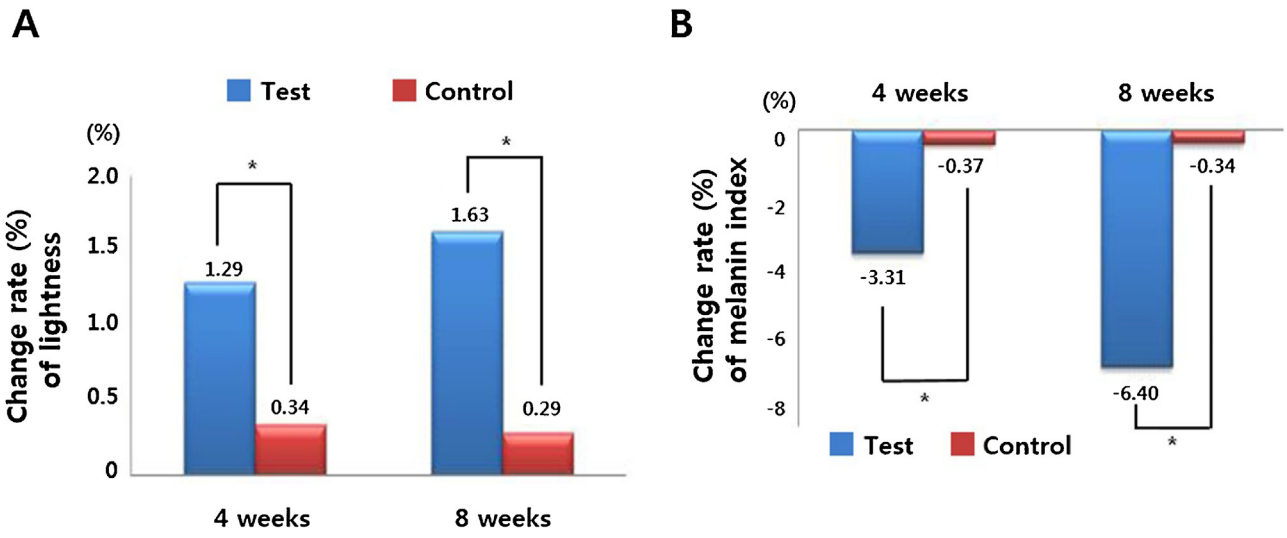

Fig. 7. Change rates in the lightness and hyperpigmentation around eyes following 8 consecutive weeks of the application of the creams. Hyperpigmented spots around eyes were analyzed after 4 and 8 weeks on lightness using chroma meter CM700d, and melanin index using mexameter MX18. Averaged change rates of the lightness (A) and melanin index (B) were calculated compared with before in the treatment of the creams. Test group (blue) was treated by 1% MADHP containing cream and control group (red) was treated by vehicle cream. Data are presented as the means ± SD (n = 21).*$p < 0.05$ versus the control group.

MITF transcription activity is very important for the increase in melanogenic proteins expression. Recently, inactivated Akt by cAMP induces the activation of the glycogen synthase kinase 3β (GSK3 β), which is inhibited its phosphorylation at serine 9, and then phosphorylates MITF at serine 289 [10]. Phosphorylated MITF binds the M-box of the promoter for the increase in the expression of melanogenic proteins containing TYR and TRPs proteins [10,45]. In this study, MAHDP showed no change in Akt phosphorylation (Fig. 3A and B). Akt is also reported to regulate the CREB activation for the increase in the MITF gene expression. However, MAHDP showed no effect on the phosphorylation of CREB (Fig. 2D). These results suggest that the decrease of MITF protein expression by MAHDP is not affected through Akt pathway-mediated MITF transcriptional activity.

MITF downregulation pathway is related with two degradation cascades; the proteasomal and endosomal/lysosomal cascades for the proteolysis of abnormal proteins [46]. Proteasome, a multi-catalytic proteinase complex, removes selectively misfolded or abnormal proteins tagged by an ubiquitin in the endoplasmic reticulum [47,48]. Lysosomes play a pivotal role in melanogenic protein degradation by its excretion of lysosomal enzymes including some glycosidases, proteases and sulfatases (ex. β-hexosaminidase and β-mannosidase) [49]. Especially, proteasome/lysosome activations are increased under the condition of excessive melanogenesis [50]. To elucidate MITF degradation whether by MAHDP mediated-proteasomal or lysosomal pathway, we pre-treated MG132 (a proteasome inhibitor) and/or chloroquine (a lysosomal proteolysis inhibitor) under the treatment of

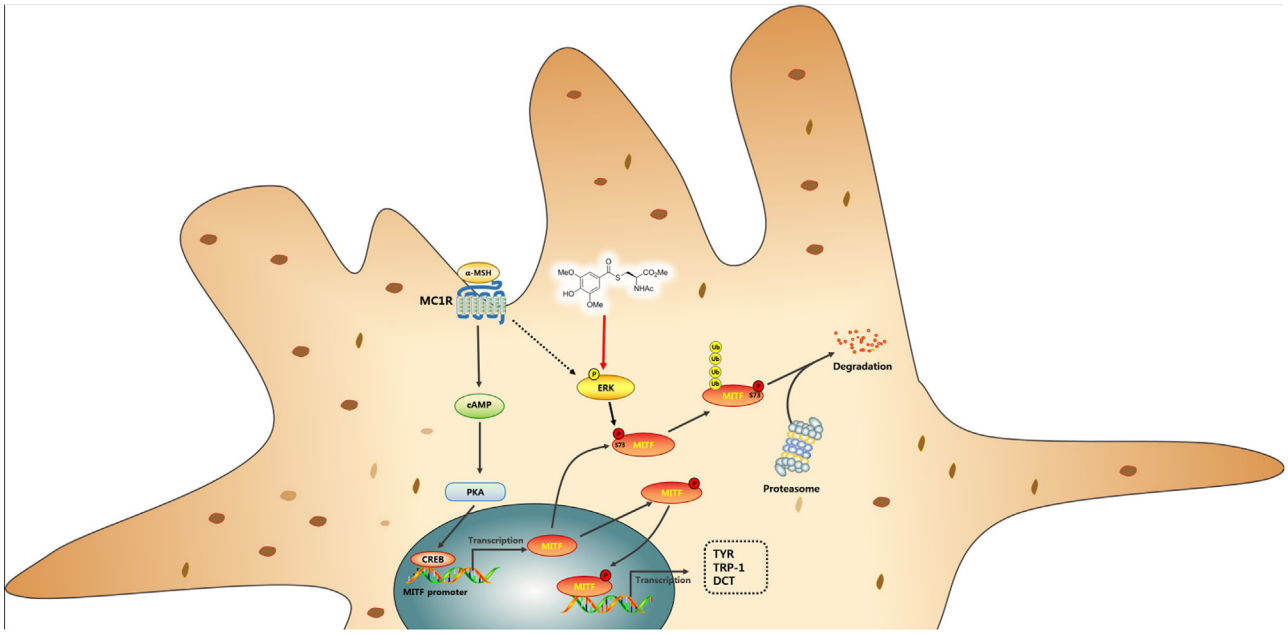

Fig. 8. Schematic presentation of the mechanism underlying inhibitory action of Methyl-2-acetylamino-3-(4-hydroxyl-3,5-dimethoxybenzoylthio)propanoate (MAHDP) on α-MSH-stimulated B16F1cells. MAHDP suppresses melanin synthesis mechanism through ERK phosphorylation, leading to phosphorylation of MITF at Serin73, and then ubiquitination for proteasomal degradation.

cycloheximide (a protein synthesis inhibitor) and MAHDP. MG132 almost completely restored MITF levels decreased by MAHDP, but chloroquine did not affect the MITF levels (Fig. 4A). Also, MAHDP indicated no effects in TYR protein in a presence of cylcoheximide. MAHDP treatment increased phosphorylation of MITF at serin 73 (Fig. 4B) and MITF ubiquitination (Fig. 4C). These results suggest that decrease in MITF protein by MAHDP is associated with the proteasomal degradation through MITF phosphorylation and ubiquitination in melanocytes.

MAPK family, including p38 MAPK, ERK and JNK, is reported to increase in mammalian melanogenesis through MITF degradation [9,51–53]. Interestingly, p38 MAPK, ERK and JNK are also reported to increase in MITF gene expression under oxidative stress [54,55]. Thus, MAPK has many issues to elucidate their roles in the regulation of melanogenesis. However, in MITF proteasomal degradation pathway, a role of ERK is well known that its active form phosphorylates MITF at serine-73 by during post-translational process, which leads to ubiquitination and the degradation by proteasome, finally decreasing the melanogenic proteins synthesis [20,56,57]. Especially, ERK cascade is known for a major signaling pathway, which can control cell growth, differentiation and MITF activity [58,59]. In this study, our data showed that MAHDP increased the ERK phosphorylation for 1 h strongly but did not affect JNK and p38 MAPK (Fig. 3A and B). Furthermore, PD98059, an ERK inhibitor, reversed MITF and TYR expression decreased by treatment of MAHDP, and decreased the phosphorylation of ERK (Fig. 3C), and subsequently increased melnanogenesis (Fig. 4C). Previous studies have also shown that ERK phosphorylation suppressed MITF expression and production of melanin through proteasomal degradation pathway in melanocytes [60]. Thus, we elucidated the depigmenting effect of MAHDP on MITF degradation via ERK phosphorylation during the progress of melanogenesis.

In summary, MAHDP inhibits melanin contents via MITF proteasomal degradation depending on proteasomal degradation via ERK phosphorylation mediated phosphorylation and ubiquitination of MITF in B16F1 cells without tyrosinase and antioxidative activities (Fig. 8). Our results suggest that the specific mechanism of MAHDP can effectively decrease melanogenesis by MITF degradation only without other depigementing effects. MAHDP is a differentiated whitening agent because almost all the agents have more than one pathway to decrease the melanogenesis in melanocytes. We also found that MAHDP can significantly decrease MITF, TYR level and melanin contents in HEM cells through MITF proteasomal degradation (Fig. 5) without concomitant changes of cell morphology. MAHDP also suppressed melanin production in UVB irradiation stimulated-reconstructed 3D human skin tissue (Fig. 6), and increased skin lightness and decreased melanin index at hyperpigmented skin around the eyes without skin irritation (Fig. 7). Hence, MAHDP could be applied as a dermatological depigmenting agent in pharmaceutical and a cosmetic ingredient for the improvement of skin hyperpigmentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2018.04.011.

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