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The inhibitory effect of egg white lysosome extract (LOE) on melanogenesis through ERK and MITF regulation

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Abstract Lysosome organelle extract (LOE) was derived from egg whites. The lysosome is an intracellular organelle that contains several hydrolysis enzymes. Previous studies have reported that LOE performs important functions, such as melanin decolorization and anti-melanin production in B16F10 melanoma cells. However, its principal molecular and cellular mechanisms have not been elucidated till date. In non-cytotoxic conditions, LOE significantly inhibited α-MSH induced melanin synthesis of murine B16F10 cells. The anti-melanogenic activity of LOE was mediated by suppressing the mRNA expression of tyrosinase enzyme, tyrosinase related protein-1/2 (TRP-1/2), and microphthalmia-associated transcription factor (MITF) genes. By performing western blot analysis, we found that LOE significantly attenuated melanogenesis. In this case, LOE helped in increasing extracellular receptor kinase (ERK) phosphorylation in α-MSH induced B16F10 cells. Furthermore, MITF is found to be a key regulatory transcription factor in melanin synthesis; it was down-regulated by LOE through ERK phosphorylation. In this experiment, PD98059 (MEK inhibitor) was used to check whether LOE directly regulated the activity of ERK. Although LOE exerted inhibitory effect on melanin synthesis, we could not observe this effect in PD98059-treated α-MSH induced B16F10. These results strongly indicate that LOE is related to ERK activation and MITF degradation in anti-skin pigmentation. Hence, LOE should be utilized as a whitening agent of skin in the near future.

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Introduction

Melanin is mainly produced in melanocytes; these cells are located in epidermis of skin. When skin is exposed to ultraviolet (UV) rays of the sun, the synthesis of melanin is promoted. Many research have proved that this is a defense mechanism to protect the skin from harmful effects of UV rays. However, a constant secretion of melanin can lead to hyperpigmentation of the skin. Many people may develop dark spots and freckles on the skin. This usually occurs whenever there is an abnormal synthesis of melanin in the skin. Furthermore, UV rays cause damage to the DNA of keratinocyte. In these conditions, alpha-melanocyte stimulating hormone (α-MSH) is secreted throughout by activating the expression of p53 protein [1,2]. When α-MSH binds with melanocortin 1 receptor (MC1R) of melanocyte, the microphthalmia associated transcription factor (MITF) is expressed through cAMP/PKA/CREB pathway [3]. In the human melanocyte, MITF is a key transcription factor in melanogenesis and its activity is regulated by multiple phosphorylation sites; it regulates the expression of various genes that cause pigmentation, and its activity is regulated when phosphorylation occurs at specific serine position of its biomolecule structure. Moreover, MITF regulates the expression of several proteins: tyrosinase, tyrosinase-related protein 1 and tyrosinase-related protein 2. These proteins are found to be involved in promoting the synthesis of melanin in melanocyte. Tyrosinase participates in the biochemical reaction that oxidizes tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and DOPA quinone. Therefore, we consider it as an important enzyme that regulates melanogenesis [4,5]. MITF activity is regulated by controlling the specific phosphorylation site and its proteolysis through ubiquitination. Thereafter, MITF activity is

down-regulated when phosphorylation occurs on serine 409 through Ras signal pathway of ribosomal S6 kinase (RSK) enzyme. In addition, Ser 73 of MITF is phosphorylated by extracellular receptor kinase (ERK), which is a type of MAPK proteins. Thus, MITF is ubiquitinated and moved to the proteasome, where it eventually undergoes degradation [6]. Meanwhile, Phosphatidylinositol 3-kinase/Akt signaling activates the expression of glycogen synthase kinase 3β (GSK3β) and the phosphorylation of serine 298. Thus, the activation of a MITF transcription factor is induced through this pathway [7,8]. Lysosomes are eukaryotic internal organelles that perform several functions, including tissue repair, pathogen defense, cell survival, and bone or tissue repair. Inside the lysosome, the activity of hydrolytic enzymes is highly controlled at low pH. It causes the decomposition of foreign substances, such as bacteria and viruses. These substances are introduced into the body of cells through endocytosis and phagocytosis [9-11]. According to a recent report, lysosome extracts from humans (HeLa cell), egg whites, and yeast (*Saccharomyces cerevisiae*) have antibacterial, anticancer, and anti-aging activities. Furthermore, a report described how the degradation of melanin was inhibited and the production of lysosome organelle extract (LOE) was promoted in egg whites [12-15]. However, the exact cellular and molecular mechanisms of LOE have not been elucidated in melanogenesis. Therefore, in this study, we tried to elucidate the whitening mechanism at the cellular and molecular level by investigating egg white LOE in α-MSH induced B16F10 melanoma cells.

Material and Methods

Chemicals and reagents

In this study, the LOE was provided by Chonbuk National University in South Korea. We used the egg white-derived LO. The egg white-derived LO and the lysis buffer (5 mM DTT, 0.1 mM PMSF, 0.1% NP-40) were mixed in a 1:1 ratio. Then, they were vortexed for 10 min, and allowed to react chemically at 4 °C for 30 min. Thereafter, centrifugation of this suspension was carried out at 4 °C for 10 min; the centrifugation machine was allowed to rotate at 13,000 rpm. Thereafter, the precipitate was collected, and LOE was obtained from the entire supernatant. After obtaining LOE from egg whites, it was quantified by performing Bradford assay. The analysis depended on the amount of enzymes present in the lysosome.

Cell culture

The murine B16F10 (CRL-6475) melanoma is a cell line that was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Then, this cell line was cultured with Dulbeco's Modified Eagle's Medium (DMEM, Welgene Biotech Ltd., Gyeongsan-si, Korea), which contained 10% fetal bovine serum (FBS, Welgene Biotech Ltd.) and 1% penicillin-streptomycin (Pen/Strep, Gibco, Waltham, MA, USA). The cell culture was maintained at 37 °C temperature and in a humidified atmosphere of 5 % CO_2.

Melanin content assay

In this experiment, B16F10 cells were cultured in log phase. Many aliquots of 1.0×10^5 cells/well were prepared in a 6-well plate and cultured at 37 °C in an atmosphere of 5% CO_2 for 24 h. Thereafter, the cells were treated with LOE (0, 5, 10, 20 mg/mL) and cultured in a medium containing 200 nM α-MSH and 10% FBS; the cell culture was kept under observation for about 72 h. The positive control was 100 μg/mL arbutin (Sigma-Aldrich, St. Louis, MO, USA) in this assay. Finally, B16F10 cells were observed under a Digital Inverted Fluorescence Microscope (Nikon, Tokyo, Japan). After treating the cell culture with trypsin/EDTA (Thermo Fisher Scientific, Waltham, MA, USA), the absorbance of the cell culture was measured at 475 nm with the help of a UV-spectrophotometer.

RNA extraction and RT-PCR

To compare mRNA levels of genes involved in melanin synthesis, we performed quantitative RT-PCR in this study. In a 6-well plate, B16F10 cells were cultured to 4.0×10^5 cells/mL for 24 h. The cell culture was maintained at 37 °C in an atmosphere of 5% CO_2. TRIzol reagent (Ambion, Thermo Fisher Scientific, Austin, TX, USA) was aliquoted at 1 mL per well, and lysis of cells was carried out to extract RNA. Using Revertra Ace Kit (Toyobo, Osaka, Japan), we performed reverse transcription and gained cDNA from RNA (1.5 μg/mL). After diluting cDNA with Tris/EDTA buffer, we performed gene amplification by using each Taqman probe (Thermo Fisher Scientific, Waltham, MA, USA) in a real-time PCR device (Applied Biosystems, Foster City, CA, USA) device. Table 1 presents the information of probes used in this study.

Protein extraction and Western Blotting

To carry out protein extraction, we added RIPA buffer (25 mM

Table 1 Gene name and assay ID number of RT-PCR

Symbol	Gene name	Assay ID
GAPDH	Glyceraldehyde-3-phophate dehydrogenase	Mm99999915_g1
MITF	Microphthalmia-associated transcription factor	Mm00434954_mL
Tyr	Tyrosinase	Mm00495817_m L
TRP1	Tyrosinase-related protein 1	Mm00453201_m L
TRP2	Tyrosinase-related protein 2	Mm01225584_m L

Table 2 Protein name and antibody used in western blot analysis

Symbol	Protein name	Assay ID
GAPDH	Glyceraldehyde-3-phophate dehydrogenase	MA5-15738 b
ERK1/2	Extracellular signal-regulated kinases	4695 a
p-ERK1/2	Phospho-extracellular signal-regulated kinases	700012 b
p38	p38 mitogen-activated protein kinase	9212 a
p-p38	Phospho-p38 mitogen-activated protein kinase	9211 a
MITF	Microphthalmia-associated transcription factor	MA5-35146 b
Goat anti-Rabbit IgG(H+L) Secondary Antibody, HRP		31460 b
Goat anti-Mouse IgG(H+L) Secondary Antibody, HRP		31430 b

a: Cell Signaling Technology
b: Invitrogen

Tris-HCl pH =7.6, 150 nM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail, and 50 mM EDTA) to the cultured cells. The cells were lysed, and they were centrifuged at 14,000 rpm and 4 °C for 15 min. The extracted proteins were mixed with β-mercaptoethanol and 4X laemmli sample buffer (31.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% bromophenol blue). Thereafter, the reaction mixture was heated to 95 °C for 5 min.Thus, electrophoresis was performed on a 10% SDS-PAGE gel at 145 V for 55 min. The protein was transferred to PVDF membrane by using an iBlot dry blotting system (Invitrogen, Waltham, MA, USA). Subsequently, it was blocked with 10% skim milk at room temperature. After reacting the primary antibody with cell culture at 4 °C, the resultant culture was washed thrice for 10 min with 1x TBST buffer. The secondary antibody was hybridized at room temperature for 1 h. Then, the antibody was washed three times for 10 min with 1x TBST buffer. Enhanced chemiluminescence solution (Bio-rad, Hercules, CA, USA) was mixed with cell culture, and the amount of protein was measured by using an image processing device (Microchemi-DNR, Neve Yamin, Israel). Finally, the expression level of proteins was analyzed by using the Image J program (N.I.H, Bethesda, MD, USA). The antibodies used in this experiment are presented in Table 2.

Statistical processing

In this study, all the experiments were repeated three or more times. The mean and the standard deviation were expressed. Using Student's t-test, a statistically significant result was ascertained. In this case, the p value was less than 0.05.

Results

The effect of LOE on the inhibition of melanogenesis

The cytotoxicity of LOE was determined by performing CCK-8 assay. On the other hand, the non-cytotoxic concentration was set to 20 mg/mL [15]. To determine the effect of LOE on the melanin inhibitory activity, we performed the melanin contents assay with B16F10 cell. In the α-MSH-treated group, melanin production increased by 268.3±6.3%. In the arbutin (100 μg/mL) group, which was used as a positive control, melanin production decreased by about 43.7±1.4%. Following the treatment of cell culture with egg white-derived LOE, the melanin production almost similarly inhibited from 5 to 20 mg/mL concentration. Thus, compared to the α-MSH-treated group, the decrease in melanin production was as much as 59.4±0.3, 62.4±0.7, and 62.0±0.3% at each LOE 5, 10, 20 mg/mL concentration, respectively (Fig. 1A). Second, we evaluated the expression of mRNA in melanogenesis-related genes by performing RT-PCR. In α-MSH-stimulated B16F10 cells, the expression level of MITF increased by about 1.4 fold, but it decreased by 85.96±0.45% in the condition of optimal LOE (20 mg/mL) (Fig. 1B). In addition, LOE significantly inhibited the expression of tyrosinase enzyme and TRP1 genes by 85.38±1.03 and 56.55±0.31%, respectively, and under the same condition (Fig. 1C, D). During melanin synthesis, LOE significantly inhibited the mRNA expression of MITF, tyrosinase enzyme, and TRP1 gene in α-MSH stimulated B16F10 cells.

The effect on ERK phosphorylation and MITF reduction by egg white-derived LOE

To elucidate the reaction mechanism of LOE at the cellular and molecular level, we treated α-MSH-stimulated B16F10 cells simultaneously with LOE (5, 10, 20 mg/mL) for two h in the presence of serum. In these conditions, α-MSH stimulated MC1R; it subsequently induced adenylate cyclase and converted ATP to cAMP in melanocytes of skin. Thereafter, cAMP finally induced the phosphorylation of CREB through protein kinase A (PKA) pathway. Moreover, CREB is activated directly by ERK of Ras pathway. Somehow, CREB phosphorylation ultimately turns on melanin production. Furthermore, LOE significantly increased the phosphorylation of ERK MAPK protein in α-MSH stimulated B16F10 cell (Fig. 2A). In particular, since the cell line was simultaneously treated with α-MSH and LOE, ERK phosphorylation increased by about 230% at LOE maximum concentration (Fig. 2B). However, no significant change was observed in the p38 MAPK pathway. Under the same conditions, LOE significantly reduced the expression level of MITF protein in converse pattern

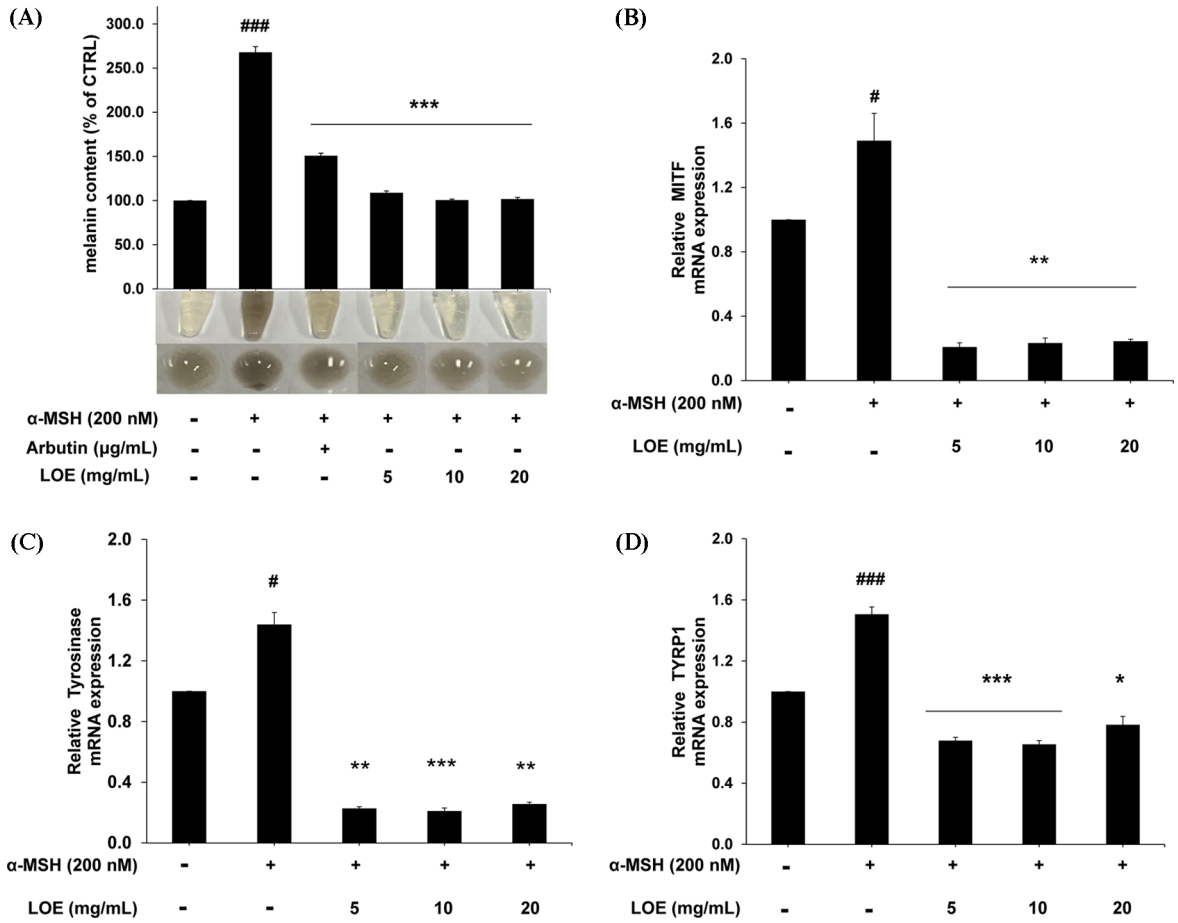

Fig. 1 The inhibition of melanin synthesis by LOE. (A) B16F10 cells were co-treated with LOE and α-MSH for 72 h. Melanogenesis was significantly reduced by LOE (20 mg/mL) at its maximum concentration. At this stage, cytotoxicity was not observed. Melanin production was significantly decreased by LOE rather than arbutin, which was the positive control in this study. (B) B16F10 cells were co-treated with LOE (5, 10 and 20 mg/mL) and α-MSH (200 nM) for 24 h. Furthermore, MITF gene expression was suppressed by LOE in the presence of α-MSH. Finally, LOE significantly decreased the expression of (C) Tyrosinase enzyme and (D) TRP1 genes under same conditions. The data were expressed as the mean ± SD. $^{\#}p$ <0.05, $^{\#\#\#}p$ <0.001, compared to the control group. *p <0.05, **p <0.01, ***p <0.001, compared to the α-MSH treated group

(Fig. 2C). Based on these results, we conclude that LOE promoted the phosphorylation of ERK. At the same time, it also suppressed the expression of MITF protein.

The inhibition of melanin synthesis by LOE is related to ERK phosphorylation

In this study, PD98059 acts as an inhibitor of ERK through MEK inactivation. We used PD98059 to determine whether LOE was directly related to Ras pathway. The murine B16F10 cells were activated with α-MSH. Both LOE and arbutin inhibited melanin synthesis normally; but PD98059 alone did not fully inhibit melanin synthesis in α-MSH stimulated B16F10. This indicates that although ERK is inhibited, melanin synthesis proceeds normally through melanogenesis of α-MSH signal. However, melanin synthesis was less suppressed in the case of α-MSH + LOE (20 mg/mL) + PD98059 group than in that of α-MSH + LOE (20 mg/mL). This indicates that PD98059 impacted the LOE's function of anti-melanogenesis (Fig. 3A). Under the conditions mentioned above, we observed that phosphorylational change of ERK was brought about by PD98059. Moreover, ERK phosphorylation was more inhibited in the case of α-MSH + LOE (20 mg/mL) + PD98059 group than in that of α-MSH + LOE (20 mg/mL) group (Fig. 3B). These results indicate that PD98059 affects the LOE's inhibitory activity on melanogenesis. Based on these results, we conclude that LOE might be directly exhibiting whitening effect through ERK phosphorylation and MITF degradation (Fig. 3C).

Discussion

A minimum exposure to UV rays is needed to maintain healthy skin in subjects. The skin tissue contains a pigment named melanin, which prevents the penetration of UV rays into human

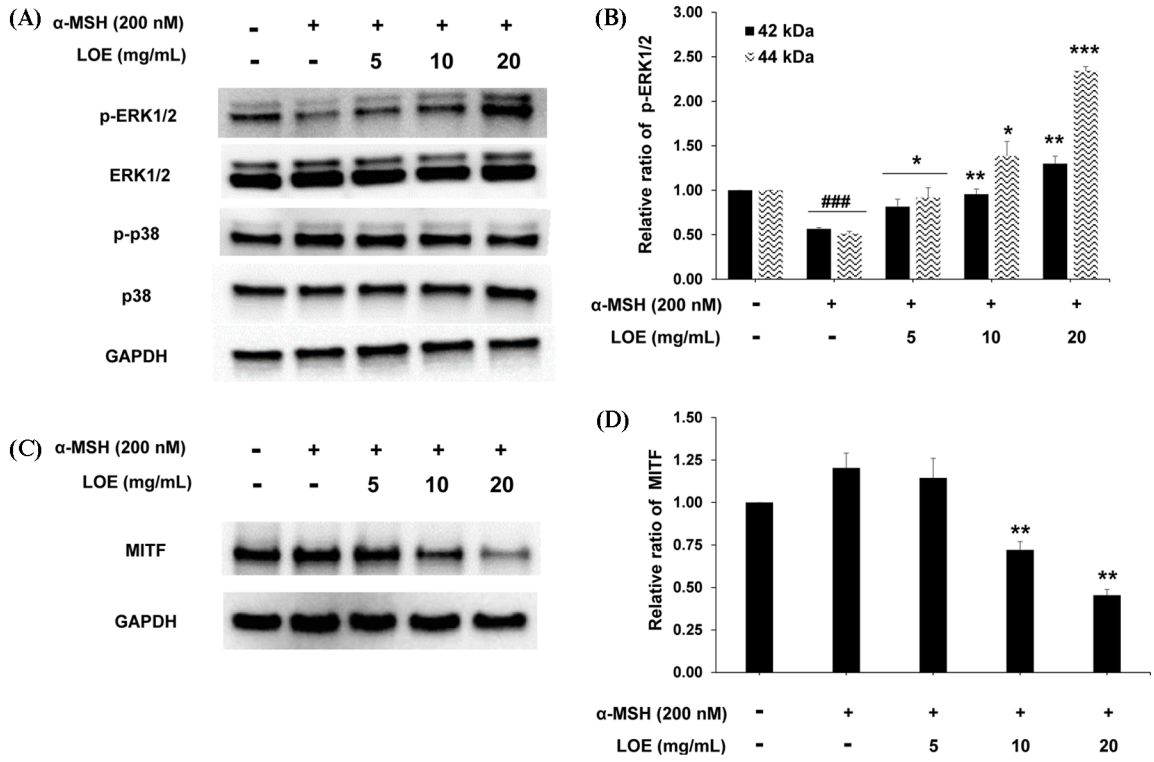

Fig. 2 In this experiment, LOE increased ERK phosphorylation in α-MSH stimulated B16F10 cells. Meanwhile, it also significantly inhibited the expression of MITF protein. The cells were co-treated with LOE and α-MSH (200 nM) for 2 h in the presence of serum. (A) The level of MAPK (ERK and p38) proteins was measured by Western blot analysis. (B) Protein density was defined as the relative ratio of p-ERK/ERK, and it was measured with Image J software. (C) The MITF protein level decreased due to LOE in a concentration dependent manner. (D) The MITF relative ratio of MITF/GAPDH. The data were expressed as the mean ± SD. $^{###}p$ <0.001, compared to the control group. *p <0.05, **p <0.01, ***p <0.001, compared to the α-MSH treated group

body. However, an excessively generated melanin rather causes pigmentation, freckles, and skin darkening [17-19]. Therefore, many studies have focused on analyzing the efficacy of whitening cosmetics, which are actively being used to improve skin discoloration. Recently, researchers have reported that whitening materials are being developed by stimulating inhibitors; these substances are involved in each step of melanin production. The primary anti-melanogenic agents were tyrosinase inhibitors; the regulators of MITF expression were associated with α-MSH, melanosome transfer inhibitors, etc [20-22]. In this study, we elucidated the function of LOE on melanogenesis. Moreover, we also explored the cellular/molecular mechanism of B16F10 cell model and its inhibitory effect on melanogenesis, which was achieved through ERK and MITF regulation. Recently, we reported that LOE induces melanin de-colorization, anti-melanin production, and wound healing in the skin of B16F10 melanoma cells [15]. When LOE was treated with non-cytotoxic concentration, it significantly inhibited melanin synthesis, the mRNA expression of MITF, and the expression of tyrosinase enzyme and TRP1 genes in α-MSH stimulated B16F10 cells (Fig. 1). Tyrosinase plays a critical or crucial role in melanogenesis by converting tyrosinase enzyme into L-DOPA and Dopaquinone [23]. Therefore, we tested whether LOE inhibits the activity of tyrosinase enzyme by performing in vitro tyrosinase assay, but there was no effect (data not shown). Overall, LOE did not directly inhibit the activity of tyrosinase enzyme, and its inhibitory activity was only observed in the mechanism of gene expression. In addition, there was no effect on PKA/CREB regulation by LOE (data not shown). However, LOE significantly increased the phosphorylation of ERK. Moreover, it inhibited the expression of MITF protein in a concentration dependent manner (Fig. 2). In this experiment, MITF was activated by GSK3β through the PI3 kinase, and Akt and mTOR pathways. Moreover, it was also degraded when phosphorylational regulation of serine 73 and serine 409 was brought about by ERK and RSK. An activated ERK directly controls the phosphorylation of serine 73 of MITF, which subsequently degrades MITF and stop the production of melanin in melanocyte [24-27]. Furthermore, PD98059 acts as an inhibitor of ERK by inactivating MEK. Herein, we added PD98059 to ascertain whether LOE was directly related to the Ras pathway. The murine B16F10 cells were activated with α-MSH. Both LOE and arbutin normally inhibited melanin synthesis in stimulated B16F10 cells. PD98059 alone group is a condition which only PD98059 was treated on B16F10 cells under conditions not stimulated by alpha-MSH hormone. Thus, treatment of only PD98059 in normal cell was no effect in melanin synthesis. Also,

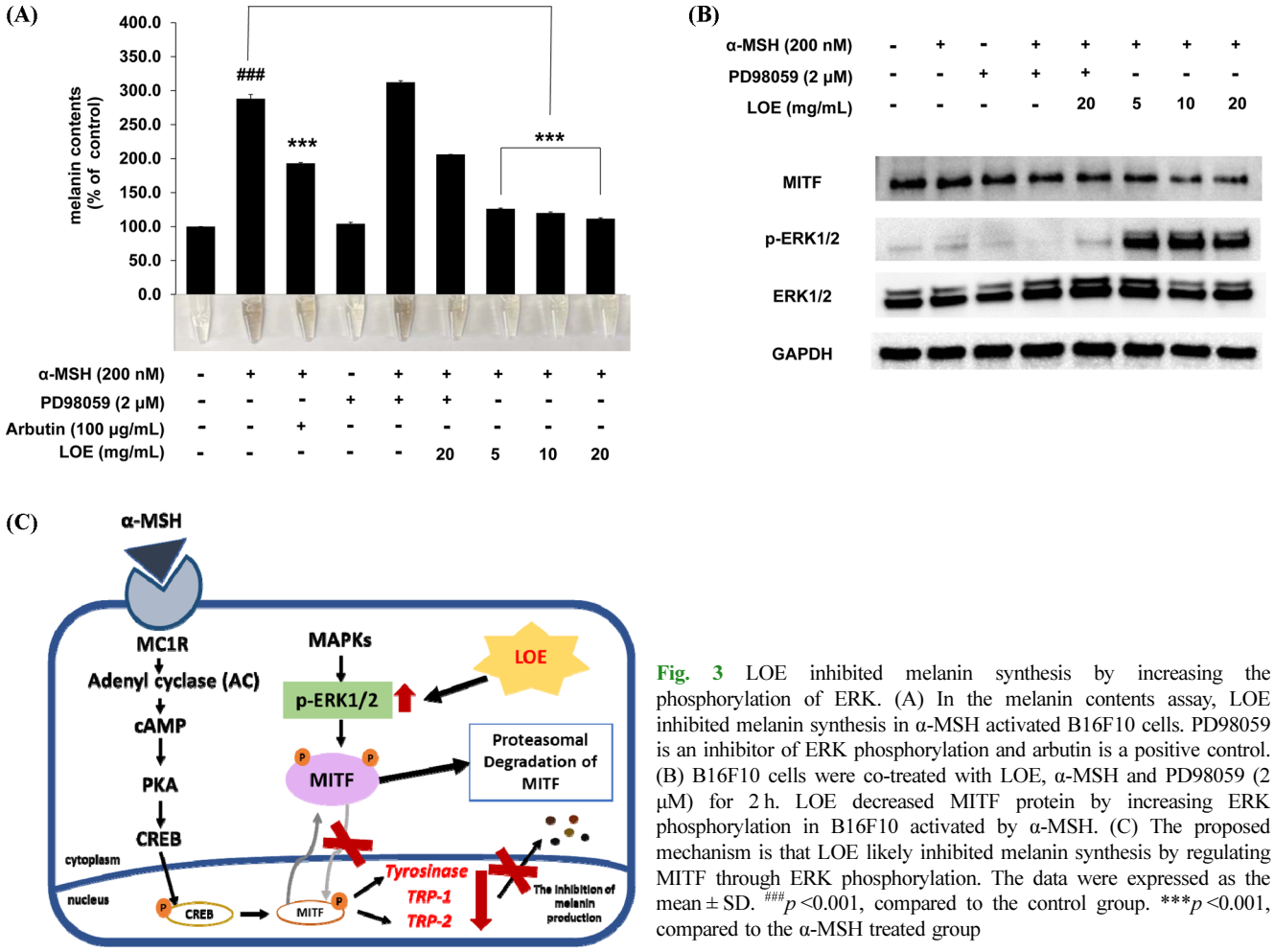

Fig. 3 LOE inhibited melanin synthesis by increasing the phosphorylation of ERK. (A) In the melanin contents assay, LOE inhibited melanin synthesis in α-MSH activated B16F10 cells. PD98059 is an inhibitor of ERK phosphorylation and arbutin is a positive control. (B) B16F10 cells were co-treated with LOE, α-MSH and PD98059 (2 μM) for 2 h. LOE decreased MITF protein by increasing ERK phosphorylation in B16F10 activated by α-MSH. (C) The proposed mechanism is that LOE likely inhibited melanin synthesis by regulating MITF through ERK phosphorylation. The data were expressed as the mean ± SD. $^{###}p$ <0.001, compared to the control group. ***p <0.001, compared to the α-MSH treated group

co-treatment of PD98059 + α-MSH was no effect in melanin synthesis, too. This implies that the role of ERK in melanogenesis is limited, but related to the MITF degradation process. As expected, in the LOE-treated group, LOE did not inhibit melanin synthesis in the presence of PD98059 (MEK/ERK inhibitor). This is because PD98059 had a negative effect on the regulated ERK activity of LOE. In summary, LOE did not inhibit melanin synthesis in PD98059-treated, α-MSH-induced B16F10 cells. Based on these results, we conclude that LOE is involved in the activation of ERK through anti-skin pigmentation process. Therefore, LOE is involved in the MITF proteolysis process. To verify the effect of LOE on protein expression of α-MSH stimulated B16F10 cells, we performed western blot assay. In this process, we found that ERK phosphorylation increased in case of α-MSH + LOE (20 mg/mL) group rather than in α-MSH + LOE (20 mg/mL) + PD98059 group. Moreover, we also found an accelerated degradation of MITF. By performing protein quantitative analysis, we estimated LOE induced phosphorylation of ERK protein. Moreover, we also witnessed the decreased expression of MITF proteins. Owing to the addition of LOE on PD98059 and α-MSH co-induced B16F10 cells, the inhibitory effect was less prominent on melanin synthesis. This trend was less prominent in the experimental group than in the α-MSH + LOE group. Finally, the phosphorylation of ERK was also less intensive due to this reaction.

Several hydrolytic enzymes are present in LOE, which is derived from egg white. Recently, we have reported that activated form of peroxidase, which is an antioxidant enzyme present in lysosomes, results in the bleaching of melanin [13,16]. Moreover, lysophosphatidic acid of eggs is known to inhibit melanin synthesis by inhibiting the protein expression of MITF and tyrosinase in α-MSH-induced Mel-Ab cell line [28-30]. It is important to note that LAMP1/LAMP2A, TRPML1, type V H + ATPase, and lipid transporters are located on the lysosomal membrane. Moreover, ERK1/2 promotes cellular proliferation through following growth factors: FGF, HGF, and SCF. Finally, ERK signals also regulate the expression of autophagy, lysosomal genes, and MITF regulation. Also, BMP4/6 interacts with BMPR1/2 on keratinocyte and melanocyte. This stimulates the proteasome-mediated degradation of MITF throughout the MAPK/ERK pathway [31]. However, more studies are needed to identify the critical factor that affects ERK phosphorylation

among LOE components, including lysosomal membrane proteins. Therefore, we would like to identify factors that continuously increase ERK phosphorylation and promote MITF degradation of lysosomal components. Nevertheless, in this study, we expect that it can be developed as a functional cosmetic material that not only has LOE's whitening activity but also improves skin health.

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