2023년 2월

석사학위 논문

Effects of berberine chloride on skin whitening and autophagy activation in cultured melanoma cells

조선대학교 대학원

글로벌바이오융합학과

류 화 정

Effects of berberine chloride on skin whitening and autophagy activation in cultured melanoma cells

배양 흑색종 세포에서 **autophagy** 활성화와
피부미백에 미치는 **berberine chloride**의 영향

2023년 **2**월 **24**일

조선대학교 대학원
글로벌바이오융합학과
류 화 정

Effects of berberine chloride on skin whitening and autophagy activation in cultured melanoma cells

지도교수 이 정 섭

이 논문을 이학 석사학위 신청 논문으로 제출함.

2022년 10월

조선대학교 대학원

글로벌바이오융합학과

류 화 정

류화정의 석사학위논문을 인준함.

위원장 조선대학교 교수 윤 성 명 (인)

위 원 조선대학교 교수 이 건 호 (인)

위 원 조선대학교 교수 이 정 섭 (인)

2022년 12월

조선대학교 대학원

CONTENTS

LIST OF FIGURES

ABSTRACT

Effects of berberine chloride on skin whitening and autophagy activation in cultured melanoma cells

Hwa Jung Ryu
Advisor : Prof. Jung Sup Lee, Ph.D.
Department of Integrative Biological Sciences
Graduate School of Chosun University

Skin is influenced by a diverse of intrinsic and extrinsic factors, including the accumulation of cellular damage, age, stress, smoking, and ultra violet (UV)-light exposure. These factors lead to skin aging associated with abnormal pigmentation, wrinkles, inflammatory response and skin cancer. Autophagy is known as a cellular self-digestion process and plays an important role in maintaining cellular homeostasis. In this process, unwanted components such as denatured proteins, damaged organelles, and pathogens are delivered to lysosome for degradation and recycling. Berberine chloride (named BBC) routinely used in this study is an isoquinoline alkaloid deprived from various plants such as barberry and goldenseal. A few studies have shown that BBC exhibits anti-viral and anti-cancer activities. However, BBC's effects on skin whitening and autophagy have not yet been clarified in detail. Therefore, the this study attempted to investigate how BBC affects skin whitening and autophagy activation in a melanoma cell line called B16-F1. The tyrosinase zymography using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate showed that tyrosine

activity could be inhibited by the BBC. The effects of BBC on the expressions of ERK and melanogenesis-related proteins (MITF, CREB, TRP-1, and tyrosinase) were also analyzed by Western blottings. As a result, BBC could increase the expressions of p-ERK, but decrease those of MITF, TRP-1, tyrosinase, CREB, and p-CREB in B16-F1 cells. In addition, BBC could decrease the melanin synthesis in B16-F1 cells in a concentration-dependent manner. These data suggest that BBC can suppress the melanin synthesis by not only inhibiting intracellular tyrosinase activity, but also by reducing the production of p-CREB and inducing MITF degradation through ERK phosphorylation, which are eventually reducing the expression of tyrosinase and TRP1. In this study, the effect of BBC on autophagy activation was also investigated. The expression levels of MAP kinases (ERK, p38, and JNK) and typical autophagic proteins (LC3, Atg12, and Beclin-1) were increased, as judged by Western blottings. These results suggest that BBC can activate the cellular autophagic system by activating MAP kinases. The effects of BBC on the autophagy activation and the melanian production were further analyzed in B16-F1 cells transfected with various small interfering RNAs (siRNAs) specific to Atg5, Beclin1, mTOR, and ERK. In the cells transfected with mTOR (negative autophagic regulator) siRNA, the synthesis of melanin pigments seemed to be increased slightly by BBC treatment, compared to that in the non-transfected group. In addition, in the cells transfected with siRNAs against Atg5, Beclin1, and ERK, the melanin synthesises could be increased significantly by BBC treatment in a concentration-dependent manner. These data clearly suggest that BBC can suppress the production of melanin by activating the autophagy system. Taken together, the results obtained by this study demonstrate that BBC can not only suppress skin aging, but also exhibit whitening effects by controlling the melanin production through the activation of the autophagic system that regulates skin cell homeostasis.

1. INTRODUCTION

Skin that consists of three layers such as epidermis, dermis, and hyperdermis forms the physical barrier to the environment and plays a variety of physiological functions (Mortiz *et al.,* 1945). At the same time, skin is inevitably affected by intrinsic factors or extrinsic factors such as hormonal changes, metabolic processes, stress, smoking, and UV light to induce skin aging (Koohgoli *et al.,* 2017). Aging skin increases the risk of skin disease such as aberrant pigmentation, inflammatory response, and skin cancer. (Kammeyer and Luiten, 2015) (Fig. 1). Melanin pigment molecules protect skin from UV light exposure, but the pigments is abnormally over-synthesized contribute to make such as melasmas, age spots and freckles occurs (Ha *et al*., 2005) (Fig. 2). In particular, photoaging caused by UV light leads to hyperpigmentation. In mammals, melanin pigments are secreted by melanocytes distributed in the epidermis (Del Marmol and Beermann, 1996). Keratinocytes produce and secrete α-melanocyte-stimulating hormone (α-MSH) by oxidative damage through UV light exposure (Fig. 2), α-MSH binds and stimulates MC1R of melanocytes (Fig. 3).

Protein kinase A (PKA) phosphorylates CREB, which controls the up-regulation of the MITF gene. MITF controls the up-regulation of melanogenesis-associated proteins, including tyrosinase and tyrosinase-related protein-1/2 (TRP-1/2). Conversely, activated ERK phosphorylates MITF and induces its degradation (Wellbrock and Arozarena, 2015)(Fig. 3). Tyrosinase acts as key enzyme to synthesize two types of melanin: eumelanin (brownish black pigments) and pheomelanin (reddish yellow pigments) in melanosomes, organelles of melanocytes (D'Mello *et al.,* 2016) (Fig. 4). Melanin is transferred from melanocytes to surrounding keratinocytes to defend the skin from UV radiation.

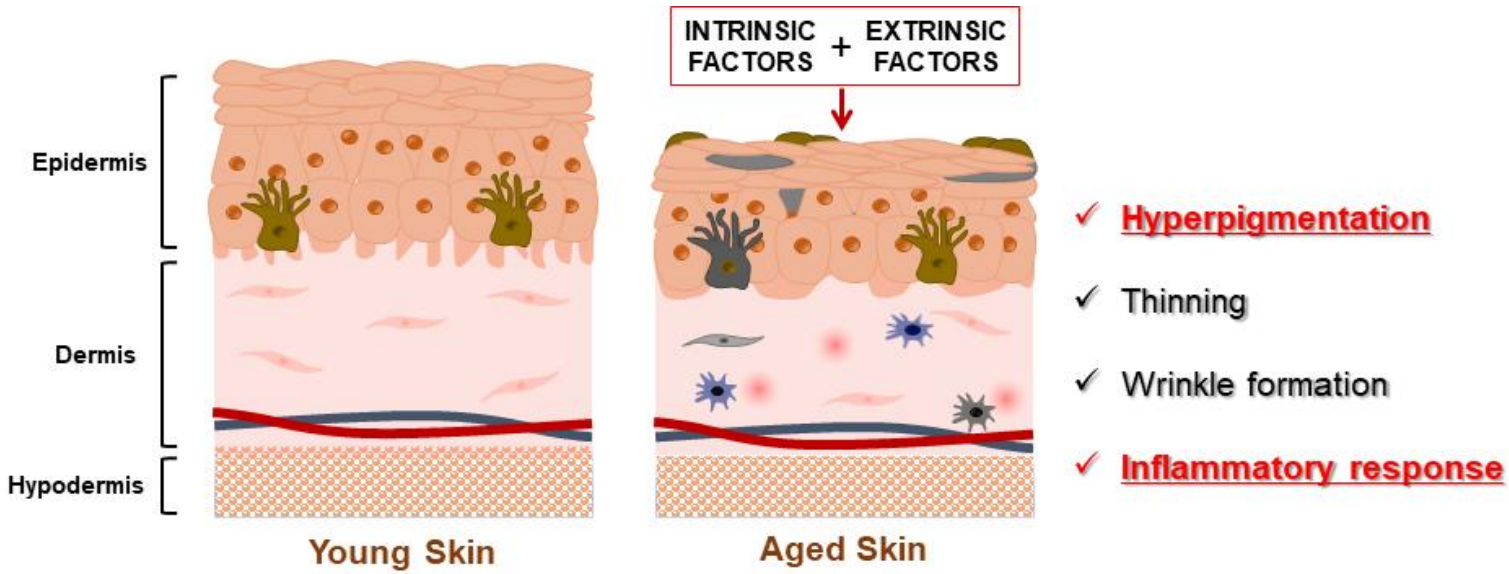

Fig. 1. Schematic diagram showing the structure of young and aged skins. Skin has three main structural layers: the epidermis, dermis, and hypodermis and then has a key protective role. Skin is affected by various of intrinsic or extrinsic factors, such as the accumulation of cellular damage, hormonal changes, UV light irradiation, and smoking to induce skin aging (Franco *et al.,* 2022). Aged skin has obvious characteristics: hyperpigmentation, thinning, wrinkles, and inflammation.

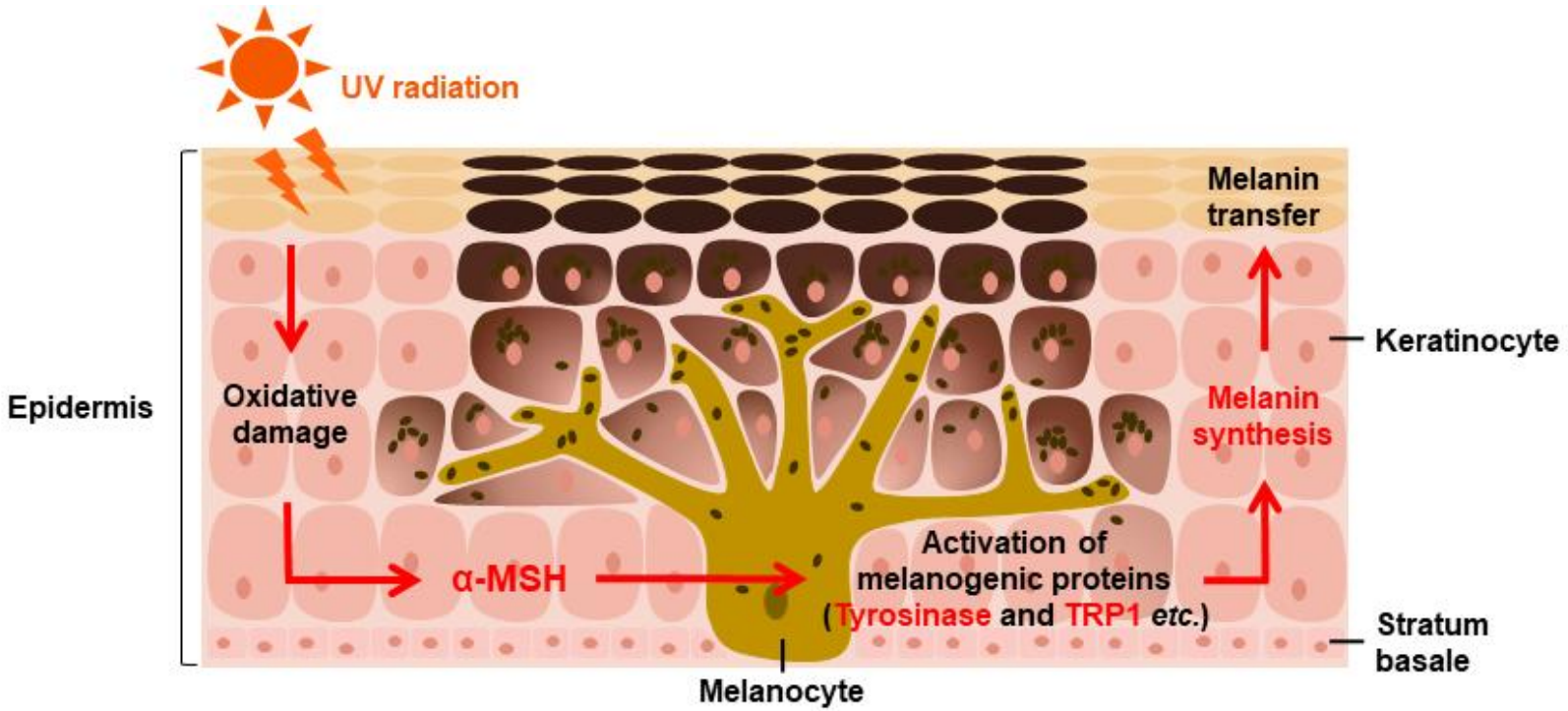

Fig. 2. Synthetic pathway of melanin pigments. Melanosomes synthesize melanin pigments in melanocytes. Following exposure to UV radiation, keratinocytes produce and secrete α-MSH. Then, α-MSH stimulates melanocytes to synthesize and store melanins. Melanin pigments are distributed to the adjacent keratinocytes (D'Mello *et al.,* 2016). Melanin pigments exhibit multifunctional biological properties, including radioprotective, anti-inflammatory, and antioxidant properties.

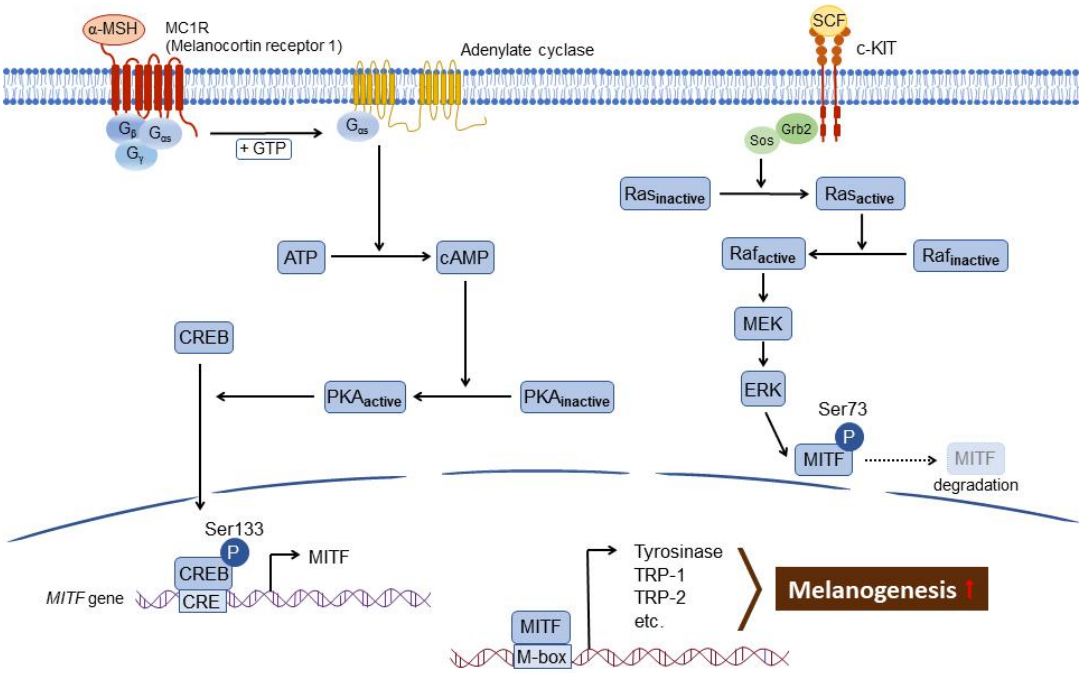

Fig. 3. Signaling pathway of melanogenesis in melanocytes. α-MSH induces melanogenesis. α-MSH binds to MC1R within the melanocyte's membrane, which leads to G protein stimulates adenylate cyclase, the enzyme that converts ATP into cAMP. Actived PKA phosphorylates CREB, which induces the up-regulation of MITF. MITF controls the expression of TRP-2, TRP-1, and tyrosinase, *etc.* Conversely, ERK inhibits melanogenesis through proteasomal degradation of MITF (Wellbrock and Arozarena, 2015).

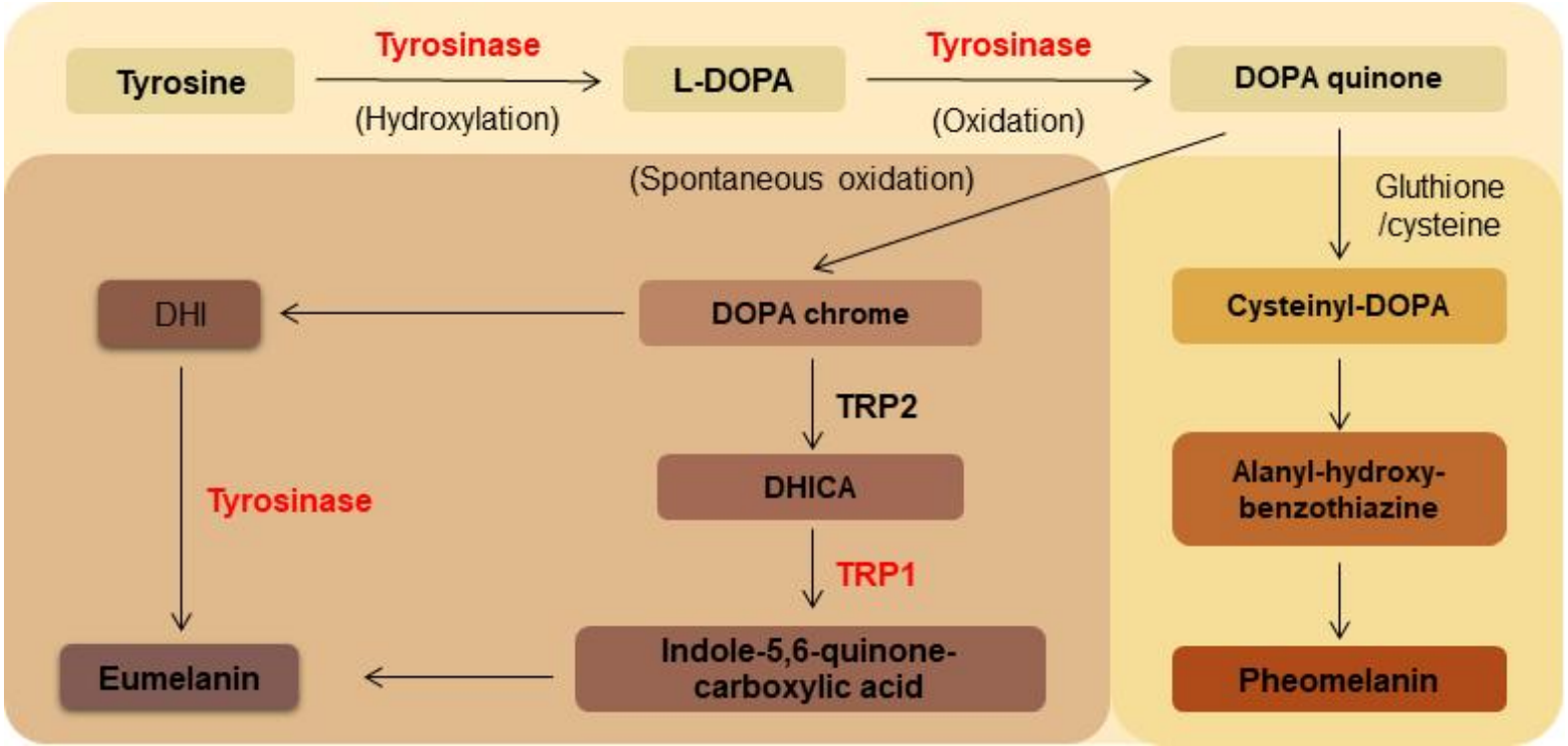

Fig. 4. Biochemical pathway of melanin synthesis. Tyrosinase, a melanogenic enzyme, acts as the main enzyme mediated by the melanin pigment production in the melanosome. Tyrosinase catalyzes the rate-limiting step of the hydroxylation of tyrosine and oxidation of L-DOPA. Two types of melanin synthesized from DOPA quinone precursors in mammals: eumelanin (brownish black pigments) and pheomelanin (reddish yellow pigments) (D'Mello *et al.,* 2016).

Autophagy plays a critical role in maintaining cellular homeostasis by inducing the degradation of cytosolic contents. Strvation induces activation of the autophagy system through the inhibition of mTOR, leading to activation of the ULK1 complex. Activated ULK1 complex and MAP kinases will phosphorylate the class Ⅲ phosphatidylinositol-3-kinase (PtdIns) to generate a local pool of phosphatidylinositol-3phosphate (PtdIns3P), which is the region of phagophore initiation (Lei and Klionsky 2021). The two ubiquitin-like conjugation systems involve in autophagy. In the first system, ATG12 is bound to ATG5 through ATG7 and ATG10. In the second system, the ATG5 and ATG12-ATG5-ATG16L1 complex involve the conjugation of LC3 to the lipid phosphatidylethanolamine (PE). The lipid conjugation leads to the conversion of LC3-I to LC3-II (Kabeya *et al*., 2004). LC3-II contributes to the closure of phagophores to form autophagosome (Fujita *et al.,* 2008). Fusion of the lysosome and the outer membrane of an autophagosome develops an autolysosome. Once the cytosolic contents are degraded, they are released into the cytosol to be reused by the cell (Karakas *et al*., 2014) (Fig 5).

Berberine chloride, an isoquinoline alkaloid is extracted from several plants (Liu *et al,* 2019). BBC was known to have many beneficial effects, including anti-viral, anti-microbial, and anti-cancer activities (Fig. 6). Recent reports showed that BBC regulates melanogenesis and autophagy system. In cultured melanoma cells, BBC inhibits the melanogeneis by down-regulating the expression of MITF and tyrosinase (Song *et al.,* 2015). Another study showed that BBC possesses the effect of autophagy activation (Wang *et al.,* 2010). Nevertheless, the exact mechanisms underlying the anti-melanogenesis properties of BBC are not fully understood, and also effects of BBC on the correlation between the skin whitening effect and autophagy have not yet been clarified. In the present study, therefore, the suppressive effects of BBC on melanin

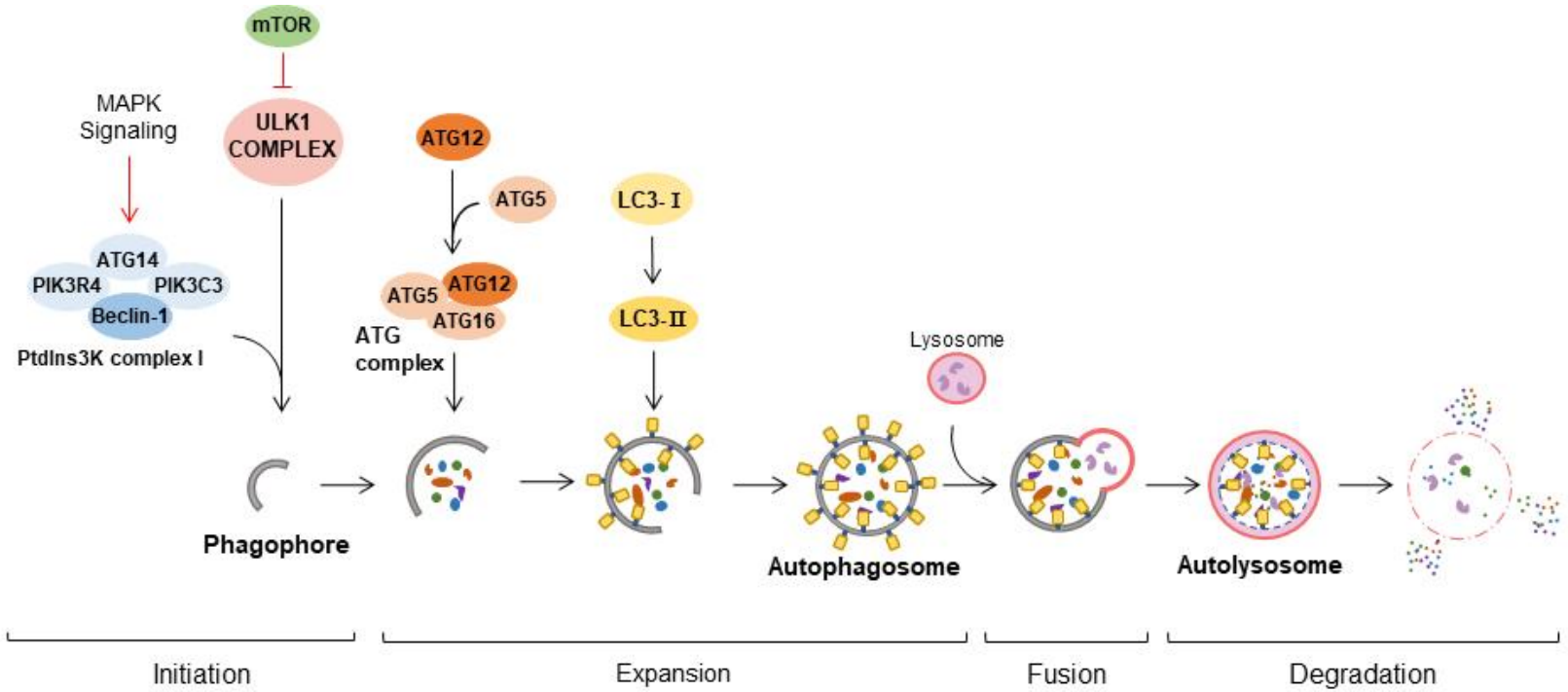

Fig. 5. Signaling pathway of autophagy. Autophagy begins to activate when mTOR, a negative autophagic regulator, is inhibited. Activated ULK1 complex and PtdIns3K complex I lead initiation of the phagophore. ATG complex expands the phagophore. The conjugation of LC3 to the phosphatidylethanolamine (PE) closes the phagophore to form an autophagosome. Fusion of lysosome and the outer membrane of an autophagosome develops an autolysosome. Vesicle contents are disposed of through a lysosomal degradation pathway, release, and reuse (Lei and Klionsky, 2021).

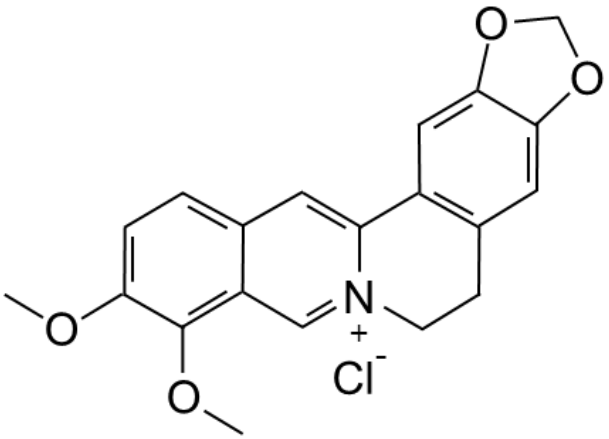

Fig. 6. Chemical structure of berberine chloride (BBC).

synthesis in terms of skin whitening and autophagy in cultured murine melanoma cell line, B16-F1.

2. MATERIALS AND METHODS

2-1. Materials

Berberine chloride (BBC) was obtained from LKT Labs (St. Paul, MN, USA). Xpert Phosphatase Inhibitor Cocktail Solution, and Xpert Protease Inhibitor Cocktail Solution were purchased from GenDEPOT (Katy, TX, USA) L-3,4-dihydroxyphenylalanine (L-DOPA), arbutin, poly-L-lysine, and α-melanocyte stimulating hormone (α-MSH) were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Transforming growth factor-β (TGF-β) was obtained from R&D systems (Minneapolis, MN, USA). Antibodies raised against tyrosinase, and tyrosinase related protin-1 (TRP-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies raised against GAPDH, Beclin-1, Autophagy related 12 (Atg12), LC3B, p44/42 MAPK, phospho-p44/42 MAPK (ERK1/2), p38 mitogen-activated protein kinases (p38 MAPK), phospho-p38 MAPK, c-Jun N-terminal kinases (JNK1), phospho-SAPK/JNK, cAMP response element-binding protein (CREB), phospho-CREB, microphthalmia-associated transcription factor (MITF), mammalian target of rapamycin (mTOR), and phospho-mTOR were obtained from Cell Signaling (Danvers, MA, USA).

2-2. Cell culture

Murine melanoma (B16-F1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagles medium (DMEM; Lonza, Swiss) containing 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS; Atlas Biologicals, USA) at 37°C in a 5% CO_2 incubator. The culture medium was changed every 2 days (Lim *et al*., 2009). The cells were harvested

through incubation of 0.5% trypsin-ethylenediaminetetraacetic acid (EDTA) (Welgen, Daegu, Korea) for 3 min when the cells were about 80% confluent.

2-3. Cell viability assay

Cell proliferation was confirmed using the CellTiter 96® AQueous Non-Radioactive cell proliferation assay (Promega, Madison, Wisconsin, USA). B16-F1 cells were cultured in 96-well plates at a density of 0.5 × 10^5 cells/well. After overnight, cells were treated with various concentrations of BBC (0, 0.1, 0.2, 0.5, 1, and 2 μg/ml) or TGF-β (0, 0.00002, 0.0002, 0.002, 0.02, and 0.2 ng/ml) for 24 h. Subsequently, 20 μl of MTS dye solution was added to each well and incubated for 4 h at 37°C in 5% CO_2. The absorption values were measured at 490 nm on a 96-well plate reader.

2-4. Tyrosinase zymography

L-DOPA staining assay was performed as previously described with slight modifications (Sato *et al.,* 2008). B16-F1 cells were cultured in 6-well plates at a density of 0.5 × 10^5 cells/well overnight. Cells were treated with various concentrations of BBC (0, 0.1, 0.2, 0.5, 1, and 2 μg/ml) in the presence of α -MSH (10 nM) for 48 h. Arbutin (0.05 mg/ml) was used as a positive control. The drug-treated cells were washed with ice-cold PBS and harvested 200 μl RIPA buffer (Biosesang, seongnam, Korea) supplemented with protease inhibitor cocktail. The cells were disrupted by freezing and thawing. The cell lysates were centrifuged at 13,000 rpm for 15 min. The protein content in the supernatant was determined by Bradford assay, with BSA as the protein standard. The proteins (100 μg) were mixed with 5X non-reducing sample buffer (no

β-mercaptoethanol or heating) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was rinsed in 10 mL of 100mM potassium phosphate buffer (ph 6.8) and equilibrated at room temperature (RT) with shaking. After 30 min, the gel was transferred to 10 mL of staining solution containing the rinse buffer supplemented with 5mM L-DOPA, and the gel was incubated for 1 h at 37 ℃ in the dark. Intracellular tyrosinase activity was visualized in the gel as a dark-colored dopaquinone band. The signal intensity of each band was quantified using the program ImageJ (National Institutes of Health, Bethesda, MD, USA).

2-5. Melanin content measurement

B16-F1 cells were cultured in 6-well plates at a density of 0.5×10^5 cells/well overnight, and then treated with BBC (0, 0.1, 0.2, 0.5, 1, and 2 μg/ml) in th presence of α-MSH (10 nM) in DMEM without phenol red (Welgene, Daegu, Korea), and further incubated for 67.5 h at 37°C in 5% CO_2. Arbutin (0.05 mg/ml) was used as a positive control. The cell-free medium was collected after centrifugation for 4 min at 13,000 rpm, and transfers to a 96-well plate. The extracellular melanin content was measured by analyzing the absorption values at 490 nm on a 96-well plate reader.

2-6. Western blot analysis

B16-F1 cells were cultured in 6-well plates at a density of 0.5×10^5 cells/well. After culturing overnight, to induce melanogenesis, the cells were

treated with α-MSH (10 nM) and then treated with BBC (1.5 μg/ml) for 24 h at 37°C in 5% CO_2. Protein samples were mixed with an equal volume of 1× SDS-PAGE sample buffer supplemented protease and phosphatase inhibitor cocktail, disrupted by sonication, denatured at 100°C for 3 min, centrifuged for 10 min at 13,000 rpm and then loaded onto 6%, 8%, 10%, 12% or 15% polyacrylamide gel. For Western blot analysis, an equal amount of each sample was subjected to SDS-PAGE and then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) using transfer buffer. The membranes were blocked with 5% skim milk in TBS-T buffer at RT for 2 h, and incubated with specific primary antibodies (1:1000 or 1:500 in the blocking buffer) at 4°C. After overnight, the membranes were washed three times for 10 min with TBS-T buffer. The membranes were then incubated at RT for 2 h with HRP-conjugated secondary antibodies (1:4000 or 1:2000 in the blocking buffer). After washing three times with TBS-T buffer, the protein expression levels were determined by analyzing the chemiluminescence substrate captured on the PVDF membrane by WESTAR SUN, WESTAR ETA C ULTRA 2.0, and WESTAR SUPERNOVA (CYANAGEN, BO, Italy). Then, the membranes were exposed on X-ray film (Fuji Film, Japan). The membranes were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2-7. Immunocytochemistry

B16-F1 cells were seeded at a density of 0.1 × 10^5 cells/well on poly-L-lysine (0.01% solution)-coated glass coverslip in 12-well plates. After incubating for 24 h, the cells were treated with BBC (1.5 μg/ml) in the presence of α-MSH (10 nM) for 15 min at 37°C in 5% CO_2. TGF-β (10 ng/ml) was used as positive control. The cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at RT. After washing cells in PBS three times,

the cells were permeabilized with 0.1% Triton X-100 for 15 min at RT and then washed with PBS for three times. The cells were incubated with blocking buffer (5% BSA in PBS) for 20 min at 4°C. After washing three times with PBS, the cells were incubated with a 1:50 dilution of anti-LC3 antibody (Santa cruz, USA) for overnight at 4°C in the dark. After incubating overnight, the cells were washed three times with PBS. The cells were incubated secondary antibody goat anti-mouse IgG-FITC (diluted 1:200 with PBS; Invirtogen, USA) for 1 h at RT in the dark. The cells were washed with PBS for three times in the dark, stained with 4'6-diamidino-2-phenylindole (DAPI; Invitrogen, USA), and observed using a Zeiss LSM 510 confocal microscope (LePecq, France).

2-8. siRNA-mediated knockdown

B16-F1 cells were cultured at a density of 0.35 × 10^5 cells/well in 100mm plates using DMEM with 10% FBS without 1% penicillin-streptomycin. After washing the cells, B16-F1 cells were transfected with 130 pmols each of mTOR, Atg5, Beclin1, and ERK small interfering RNA (siRNA; Bioneer, KOREA) with siRNA transfection reagent and suspended in siRNA transfection medium (serum-free medium) at 37°C in 5% CO_2 for 7 h. Control and transfected cells were used as described previously (Rai *et al*., 2013). After transfecting for 5 h, the media was changed and replaced with DMEM containing 10% FBS, 1% penicillin-streptomycin and incubation at 37°C in 5% CO_2. Thereafter, siRNA transfected cells were cultured in 48-well plates 0.1 × 10^5 cells/well and then treated with BBC (0, 0.1, 0.2, 0.5, 1, and 2 μg/ml) in the presence of α-MSH (10 nM). The cell-free medium transfers to a 96-well plate, and then the extracellular melanin content was measured by analyzing the absorption values at 490 nm on a 96-well plate reader.

3. RESULTS AND DISCUSSION

3-1. Effects of BBC on cytotoxicity in murine melanoma cell line (B16-F1)

To observe the possible cytotoxicity of BBC on B16-F1 cells, MTS assay was performed in B16-F1 cells after treatments with BBC (0, 0.5, 1, 1.25, 1.5, and 2 μg/ml) for 24 h. TGF-β was used as a positive control. As shown in Fig. 7, BBC did not show significant cellular toxicity up to 2 μg/ml, compared to the non-treated control. These result suggest that BBC has no cytotoxicity on B16-F1 cells.

3-2. Inhibitory effects of BBC on intracellular tyrosinase activity

It has been known that tyrosinase acts as the rate-limiting enzyme that can regulate melanin pigment synthesis (Hearing and Tsukamoto, 1991). DOPA quinone precursor, produced through the catalytic action of tyrosinase, is converted to two types of melanin, such as numelanin and pheomelanin (Del Marmol and Beermann, 1996). The effect of BBC on intracellular tyrosinase activity in B16-F1 cells was analyzed by tyrosinase zymography using L-DOPA as a substrate on 10% SDS-PAGE gels. It shown in Fig. 8, intracellular tyrosinase activity could be reduced by BBC (1.5 μg/ml) to approximately 66%, compared to those treated with only α-MSH.

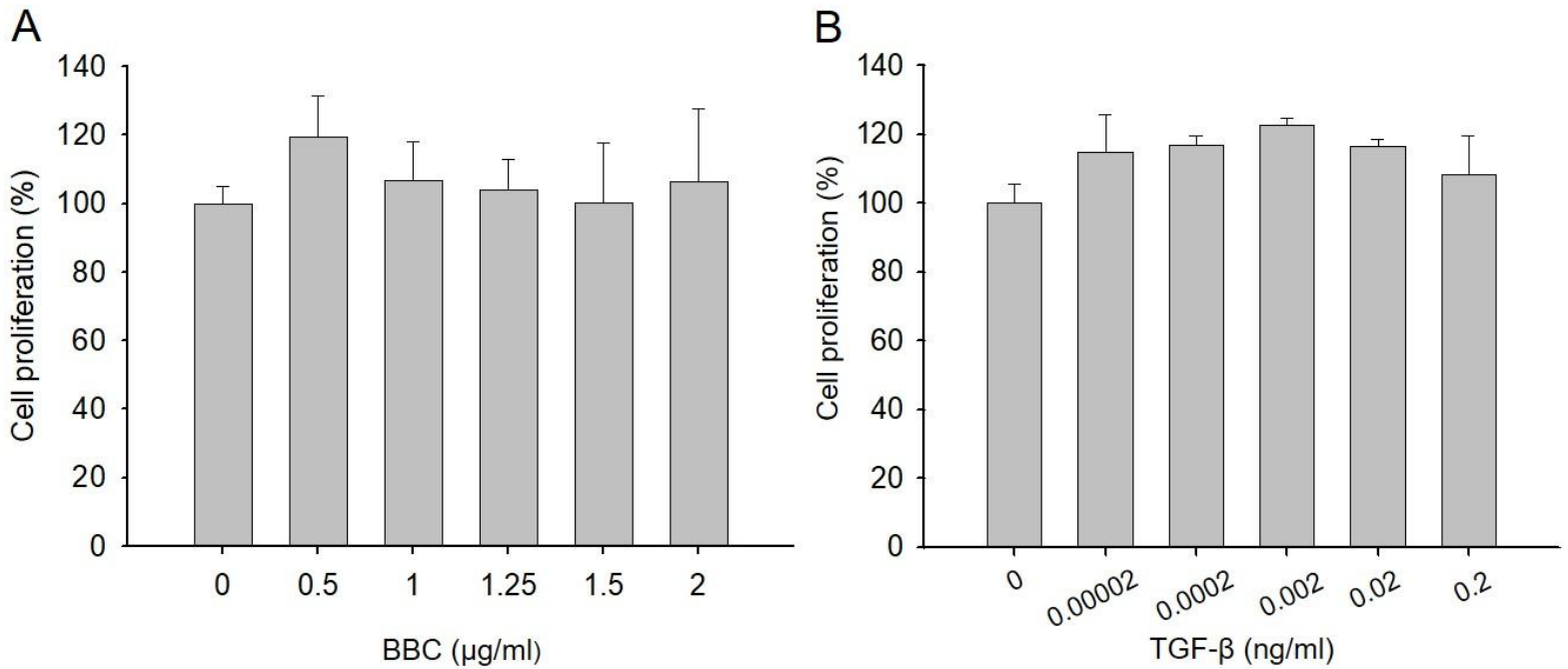

Figure 7. Effects of BBC on cytotoxicity in murine melanoma cell line (B16-F1). Cell proliferation was determined by the MTS assay as described in Materials and Methods, in which B16-F1 cells were treated with BBC (0 ~ 2 μg/ml) (A) and TGF-β (0 ~ 0.2 ng/ml) (B) for 24 h. TGF-β was used as a positive control.

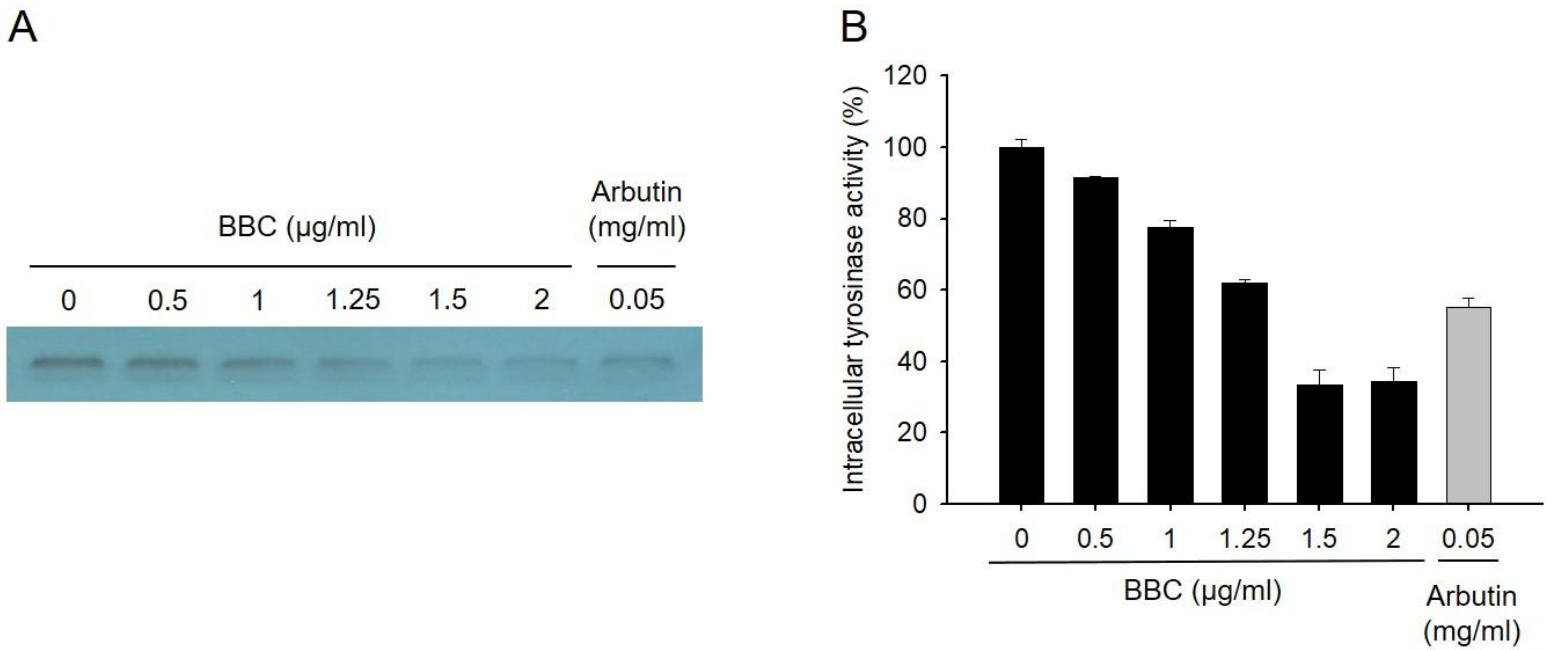

Figure 8. Inhibitory effects of BBC on intracellular tyrosinase activity. (A) B16-F1 cells were treated with BBC (0 ~ 2 μg/ml) in the presence of α-MSH (10 nM) for 48 h. After quantifying protein amounts, the intracellular tyrosinase activity was examined by tyrosinase zymography. (B) Histograms showing the relative band intensity.

3-3. Effects of BBC on ERK pathway and various melanogenesis-associated proteins in B16-F1 cells

Melanogenesis is induced by binding of α-MSH to MC1R, the receptor of melanocytes. When α-MSH attached to MC1R in melanocytes, CREB is phosphorylated by PKA signaling. p-CREB controls the expression of the MITF gene, which leads to up-regulation of key enzymes of melanin production such as tyrosinase and TRP1. Whereas it has been known that ERK inhibits melanogenesis through inducing degradation of MITF (Wellbrock and Arozarena, 2015). In this study, to investigate the inhibition effect of BBC to the expression levels of melanogenesis-related proteins were confirmed using Western blottings with the corresponding antibodies. As shown in Fig. 9, the expression levels of p-ERK is up-regulated by 120%, but the protein expression levels of p-CREB, CREB, MITF, tyrosinase, and TRR-1 were down-regulated by 18%, 28%, 39%, 46%, and 31%. In the B16-F1 cells treated with BBC (1.5 μg/ml) for 24 h, compared to those treated with only α-MSH. These data suggest that the suppressive effects of BBC on melanin synthesis are through inducing the degradation of MITF by the ERK pathway and down-regulating the expression of melanogenesis-related proteins.

3-4. Inhibitory effects of BBC on melanin synthesis in B16-F1 cells

To examine the suppressive effect of BBC on melanin synthesis, B16-F1 cells were treated to BBC (0, 0.5, 1, 1.25, 1.5, and 2 μg/ml) with α-MSH (10 nM) for 67.5 h. As shown in Fig. 10, melanin pigments in the media could be decreased by treatment with BBC in a concentration-dependent manner.

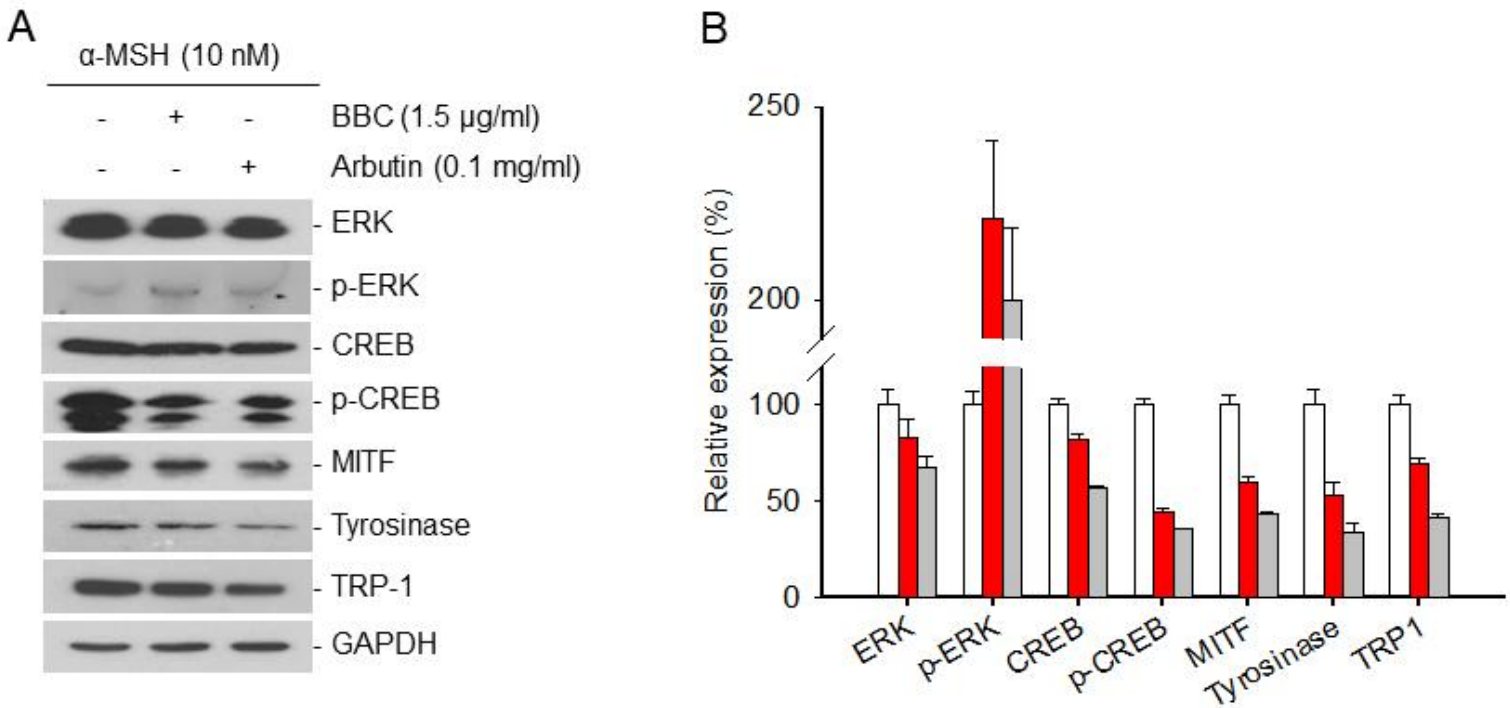

Fig. 9. Effects of BBC on ERK pathway and various melanogenesis-associated proteins in B16-F1 cells. (A) B16-F1 cells were treated with BBC (1.5 μg/ml) with α-MSH (10 nM) for 24 h. In this experiment, Arbutin (0.05 mg/ml) was used as a positive control. (B) Histograms showing the relative rates of expressions.

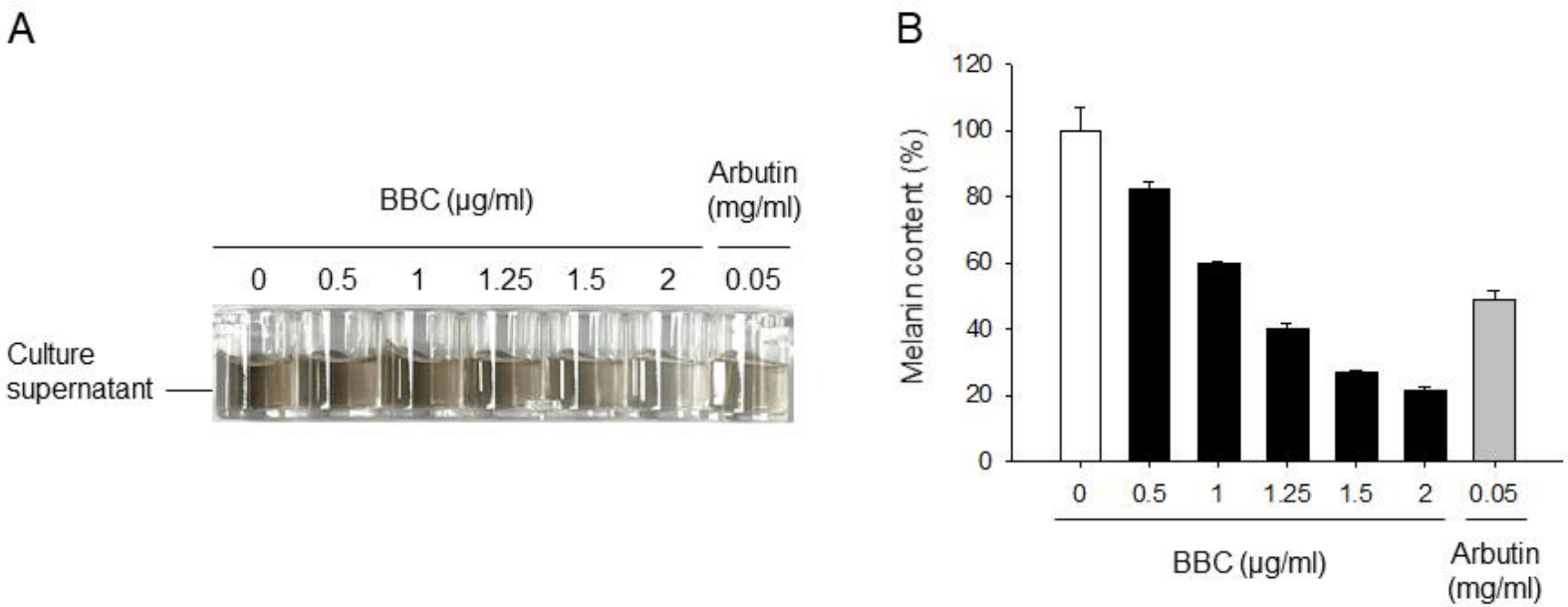

Figure 10. Inhibitory effects of BBC on melanin synthesis in B16-F1 cells. (A) Photographs showing the suppressive effects of BBC on the production of melanin pigments. B16-F1 cells were treated with BBC (0 ~ 2 μg/ml) and Arbutin (0.05 mg/ml, as a positive control) in the presence of α-MSH (10 nM) for 67.5 h. Culture supernatants were collected and photographed. (B) The amounts of melanin were determined by measuring the absorbance at 490 nm.

Melanin production was decreased by approximately 73% in B16-F1 cells treated with BBC (1.5 μg/ml), compared to that of only α-MSH. These data suggest that BBC can suppress production of melanin pigments in B16-F1 cells.

3-5. Effects of BBC on MAPK pathway in B16-F1 cells

The MAP kinases have been known to function in a variety of cellular processes including proliferation, cell differentiation, survival, and death. There are three main families of MAP kinases including ERK, p38, and JNK that respectively control the autophagy system. Activation of ERK and JNK up-regulated Beclin1 expression through induction of Bcl-2 phosphorylation. p38 increased the expression of Atg5 and Beclin1 (Sridharan *et al.,* 2011). Based on these data, the effect of BBC on the activation of MAP kinase was confirmed in B16-F1 cells. As shown in Fig. 11, when B16-F1 cells were treated with BBC (1.5 μg/ml) or TGF-β (10 ng/ml) for 30 min, the expression levels of p-ERK, p-p38, and p-JNK were up-regulated to approximately 37%, 20%, and 59%. Respectively, these results suggest that BBC induces the up-regulation of the autophagy system by activating the MAP kinases pathway in B16-F1 cells.

3-6. Effect of BBC on autophagy system in B16-F1 cells

Autophagy is a cellular self-digestion process for cellular homeostasis through the degradation of pathogens, dysfunctional proteins, and damaged organelles (Kim *et al.,* 2021). Autophagy can be divided into four steps: initiation, expansion, fusion, and degradation. Inhibition of mTOR, a critical

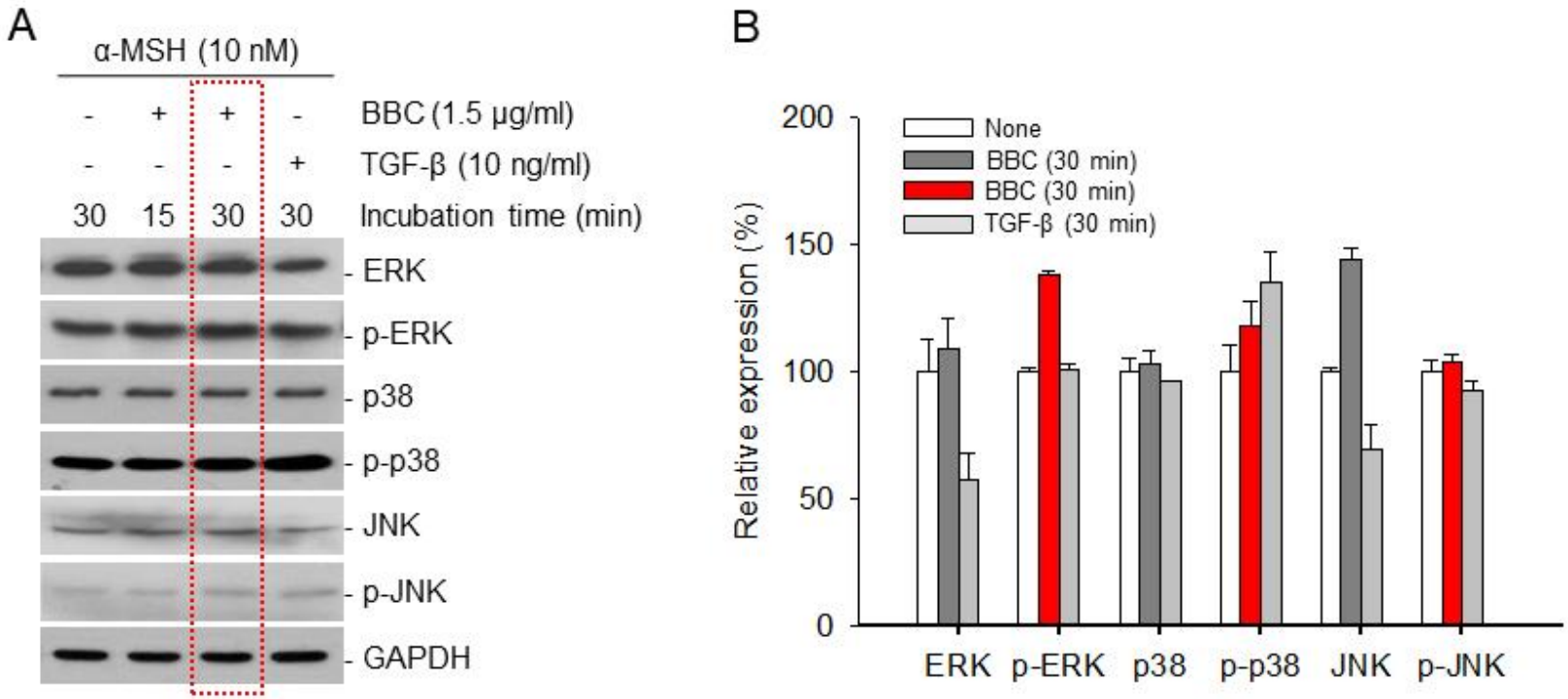

Fig. 11. Effects of BBC on MAPK pathway in B16-F1 cells. (A) B16-F1 cells were treated with BBC (1.5 μg/ml) in the presence of α-MSH (10 nM) for 15 or 30 min. In this experiment, TGF-β (10 ng/ml) was used as a positive control. (B) Histograms showing the relative expression rates. Each value was calculated as the ratio of signal intensity, compared to that of GAPDH.

negative regulator of autophagy, leads to activation of the autophagy system. Based on these data, the effect of BBC on the activation of the autophagic system was confirmed in B16-F1 cells. When B16-F1 cells were treated with BBC (1.5 μg/ml) or TGF-β (10 ng/㎖) for 30 min, the protein expression levels of mTOR and p-mTOR were suppressed to about 12% and 19%, respectively. However, the protein expression level of Beclin-1, Atg12, and LC3 was up-regulated to about 49%, 99%, and 12% by BBC (Fig. 12). These data suggest that BBC can lead to the up-regulation of autophagy-associated proteins expression in B16-F1 cells.

3-7. Immunocytochemistry analysis of the induction of autophagy by BBC in B16-F1 cells

The autophagosome, double-membrane, is the key structure in autophagy. LC3, an autophagosome marker, binds to PE within phagophores's membrane to form autophagosomes. The autophagosome captures the cytosolic contents and degrades the contents by fusion with the lysosome (Lee *et al.,* 2022). To investigate the formation of autophagosomes through autophagy activation by BBC, the LC3 expression was examined by immunohistochemistry. As shown in Fig. 13, the formation of autophagosomes increased when BBC (1.5 μg/ml) was treated with or without α-MSH.

3-8. Effects of BBC on the production of melanin synthesis in B16-F1 cells transfected with siRNAs specific to autophagy-related genes.

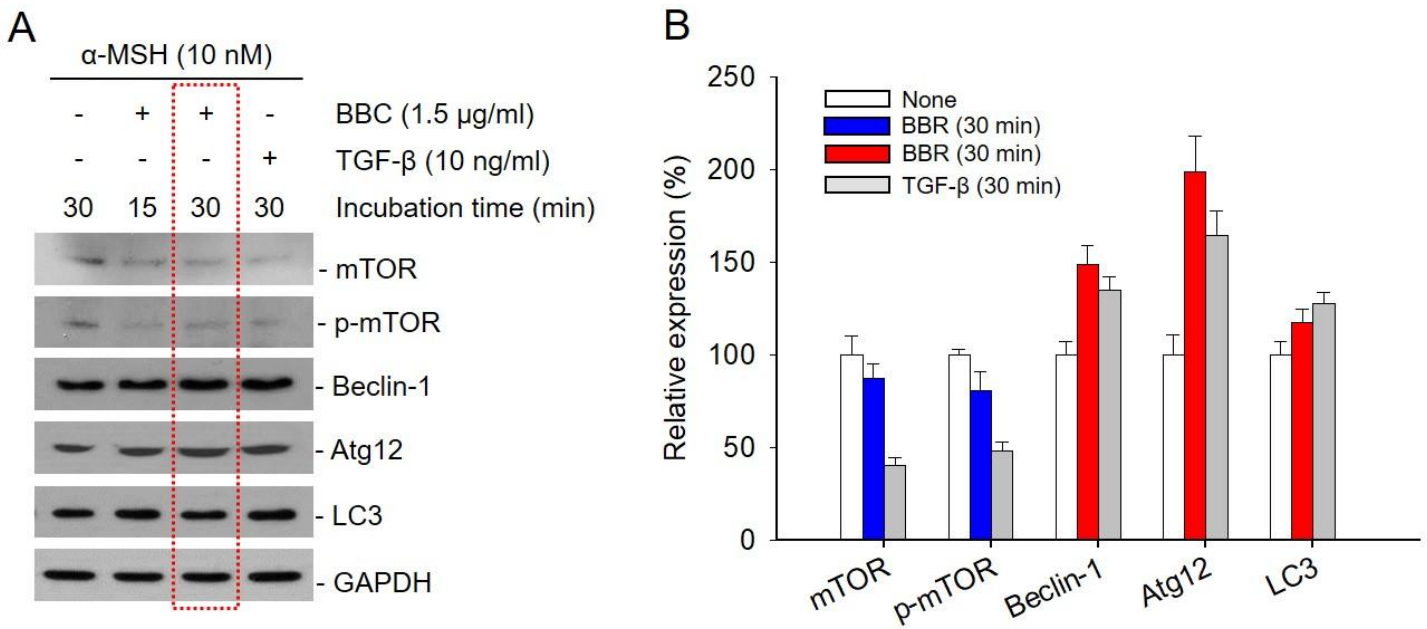

Fig. 12. Effect of BBC on autophagy system in B16-F1 cells. (A) B16-F1 cells were treated with BBC (1.5 μg/ml) with α-MSH (10 nM) for 15 or 30 min. In this experiment, TGF-β (10 ng/ml) was used as a positive control. (B) Histograms showing the relative expression rates, in which each value was calculated as the ratio of signal intensity, compared to that of GAPDH.

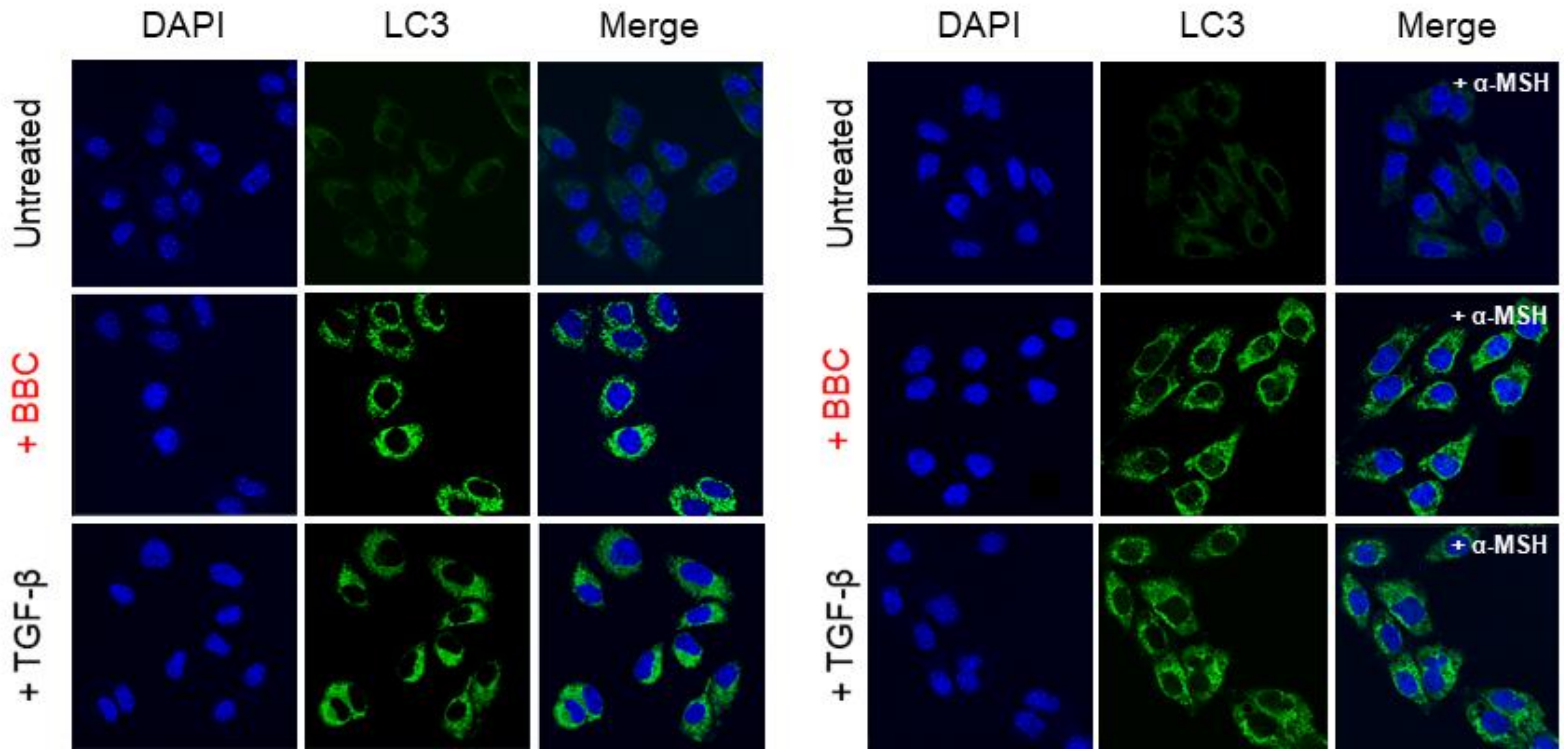

Fig. 13. Immunocytochemistry analysis of the induction of autophagy by BBC in B16-F1 cells. Confocal microscopy images show autophagosome formation in cells treated with BBC (1.5 μg/ml) or TGF-β (10 ng/ml) for 15 min or 30 min, respectively. (A) without α-MSH (10 nM) or (B) with α-MSH (10 nM). The B16-F1 cells were immunostained with anti-LC3 antibody labeled with green fluorescence. TGF-β was used as a positive control.

All the results demonstrate that BBC could inhibit melanogenesis by down-regulating the melanogenesis-related proteins and phosphorylating ERK, and also activate the autophagic system by up-regulating the autophagy-related proteins. Nevertheless, it was still necessary to determine that BBC can activate autophagic system, which leads to the inhibition of melanin production. To investigate the effects of BBC on the relationship between the inhibition of melanin synthesis and the activation of an autophagic system, reverse genetic approaches using small interfering RNAs (siRNAs) against mTOR, ERK, Beclin1, and Atg5 mRNAs were performed. When non-transfected B16-F1 cells were treated with BBC (0, 0.5, 1, 1.25, 1.5 and 2 μg/ml) for 70.5 h, there were decreases in melanin production in a concentration-dependent manner. When cells were transfected with the siRNA specific to mTOR (negative autophagic regulator), the production of melanin pigments seemed to be slightly increased by BBC treatment compared to that in non-transfected cells. In addition, the melanin synthesis could be significantly increased by BBC treatment in the cells transfected with the siRNAs specific to Atg5, Beclin1, and ERK (Figs. 14A and B). These results suggest that BBC can suppress melanin pigment production in melanoma cells through autophagy activation.

4. Conclusion

The results obtained by this study show that BBC has various effects on skin aging and whitening as follows: 1, BBC inhibits the expression of mTOR and activates MAPK pathway, resulting in up-regulation autophagy-related proteins, such as Beclin1, Atg12, and LC3, which activating the autophagy system; 2, BBC activates the ERK pathway. Phosphorylated ERK induces the degradation of MITF, which in turn down-regulates the expression of

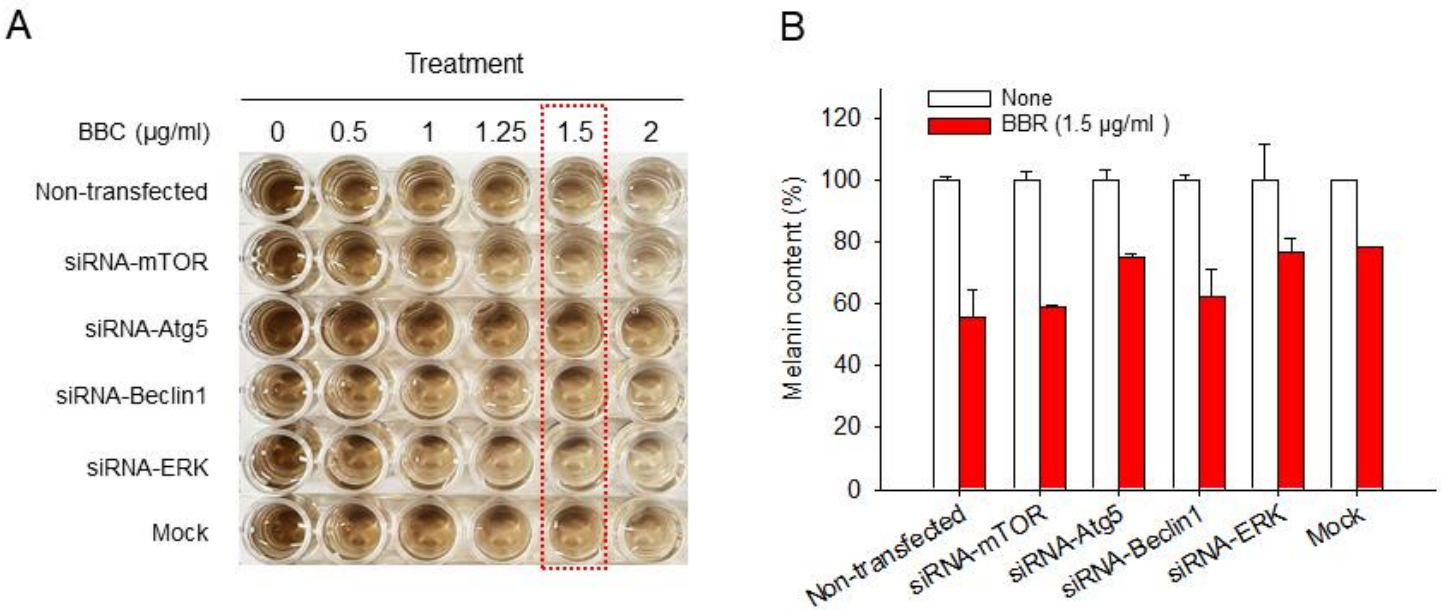

Figure 14. Effects of BBC on the production of melanin synthesis in B16-F1 cells transfected with siRNAs specific to autophagy-related genes. (A) B16-F1 cells were treated with BBC (0 ~ 2 μg/ml) after being transfected with siRNAs specific to Atg5, Beclin1, ERK, mTOR, or non-specific control (Mock). Photographs showing the inhibitory effects of the corresponding siRNAs melanin production. (B) The melanin contents were determined by measuring the absorbance at 490 nm from BBC-treatment group for 70.5 h.

tyrosinase and TRP1; 3, BBC exerts a suppressive role in the production of melanogenesis- associated proteins such as p-CREB, MITF, TRP1, and tyrosinase. These results clearly demonstrate that BBC can control the synthesis of melanin pigments in B16-F1 cells by activating the autophagy system. In conclusion, BBC could not only suppress skin aging but also exhibit whitening effects by controlling the melanin production through the activation of the autophagy system that regulates skin cell homeostasis (Fig. 15).

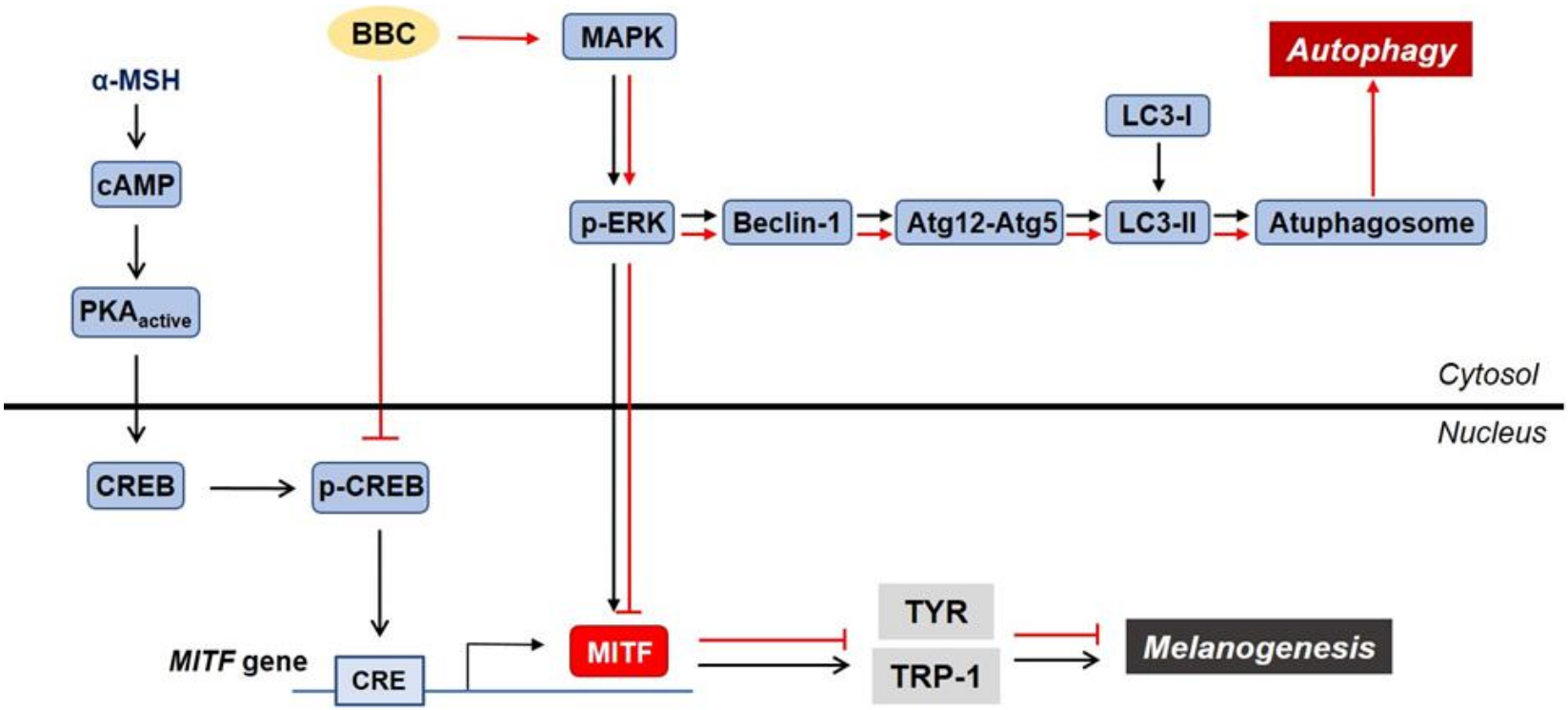

Figure 15. Summary of possible inhibitory effects of BBC on melanogenesis. BBC activates the ERK pathway, which leads to the activation of the autophagy system. BBC inhibits melanogenesis by activating ERK to induce the degradation of MITF and also suppress the activation of CREB, leading to down-regulates the expression of melanogenesis-related proteins. The signaling pathway pathways that are triggered by BBC and already known are shown in red- and gray-colored arrows.

4. 적 요

배양 흑색종 세포에서 autophagy 활성화와 피부미백에 미치는 berberine chloride의 영향

류 화 정
지도교수 : 이 정 섭
조선대학교 대학원
글로벌바이오융합학과

피부는 나이, 호르몬 변화, 스트레스, 흡연, 자외선 노출을 포함한 다양한 내재적 그리고 외재적 요소들로부터 영향을 받는다. 이러한 요소들은 비정상적인 색소침착, 주름, 염증 반응 및 피부암과 관련된 피부 노화를 유도한다. Autophagy는 기능을 다한 단백질, 세포소기관 및 병원체 등, 세포 내 불필요 구성 요소들이 리소좀(lysosome)에서 분해되어 재활용되게 하는 세포 내 소화과정이며, 세포의 항상성 유지에 매우 중요한 역할을 한다. 본 연구에 사용된 berberine chloride(BBC로 명명함)는 매자나무 및 황련과 같은 다양한 식물에 존재하는 이소퀴놀린 알칼로이드(isoquinoline alkaloid)이며, 이 화합물은 항멜라닌생성(anti-melanogenesis), 항염증(anti-inflammatory) 및 항바이러스(anti-viral) 등과 같은 다양한 효과를 지니는 것으로 알려져 있다. 그러나 피부미백과 autophagy에 미치는 BBC의 영향은 자세히 연구되지 않았다. 따라서 본 연구에서는 흑색종 세포주인 B16-F1 세포에서 BBC가 피부미백 및 autophagy 활성화에 미치는 영향을 규명하고자 하였다. L-DOPA(L-3,4-dihydroxyphenylalanine)를 기질로 tyrosinase zymography를 수행한 결과, 세포내 tyrosinase 활성이 BBC에 의해 억제됨을 확인하였다. 또한 Western blotting으로 BBC에 의한 MAP kinase(ERK)와 멜라닌 생성 관련 단백질들(CREB, MITF, Tyrosinase, TRP-1 등)의 발현양상을 분석하였다. 그 결과, BBC는 B16-F1 세포에서 p-p38, p-ERK, p-JNK의 발현을 증가시켰으나, CREB, p-CREB, MITF, tyrosinase, TRP-1 등의 발현은 감소시킴을 확인하였다. 또한 B16-F1 세포에

BBC를 67.5시간 처리하였을 때, 멜라닌 생성이 감소하는 것을 확인하였다. 이러한 결과들은 BBC가 세포 내 tyrosinase 활성을 직접적으로 저해할 뿐만 아니라, p-CREB의 생성을 감소시키고, ERK 인산화를 통해 MITF 분해를 유도함으로써 tyrosinase와 TRP1의 발현을 감소시켜 멜라닌 합성을 억제시킬 수 있음을 시사하는 것이다. 본 연구에서는 또한 autophagy 활성화에 미치는 BBC의 영향을 조사하였다. B16-F1 세포에 BBC를 처리한 후, autophagy 유도 단백질들의 발현 양상을 Western blotting으로 분석한 결과, MAP kinase(p38, ERK 및 JNK), Beclin-1, Atg12 및 LC3 발현이 증가됨을 확인하였다. 이러한 결과는 BBC가 MAP kinase들의 활성화를 통해 autophagy system을 활성화시킬 수 있음을 시사하는 것이다. 또한 autophagy 활성화와 멜라닌 생성에 미치는 BBC의 영향을 mTOR, Atg5, Beclin1 및 ERK에 대해 특이적인 small interfering RNA(siRNA)를 주입한 B16-F1 세포에서 분석하였다. 그 결과, autophagy system의 음성 조절자(negative regulator)인 mTOR에 대한 siRNA를 도입한 세포에서는 도입하지 않은 세포에 비해 BBC에 의해서 멜라닌 합성이 약간 증가함을 확인하였다. 반면, 양성 조절자(positive regulator)들인 Atg5, Beclin1 및 ERK에 대한 siRNA를 도입한 세포에서는 BBC에 의해 멜라닌 합성 상당히 증가함을 확인하였다. 이러한 결과는 BBC가 autophagy system을 활성화시킴으로써 멜라닌 합성을 억제시킬 수 있음을 시사하는 것이다. 결론적으로 본 연구에서 얻은 연구결과들은 BBC가 피부세포의 항상성을 조절하는 autophagy 시스템의 활성화를 통해 멜라닌 생성을 제어함으로써 피부의 노화를 억제할 수 있을 뿐만 아니라 미백효과를 발휘할 수 있을 가능성을 시사하는 것이다.

5. REFERENCES

Anderson, R. R., and Parrish, J. A. (1981). The optics of human skin. *J. Invest. Dermatol.* 77(1): 13-19.

Cho, Y. H., Park, J. E., and Lee, J. S. (2017). Tranexamic acid inhibits melanogenesis by activating the autophagy system in cultured melanoma cells. *J. Dermatol. Sci.* 88(1): 96-102.

D'Mello, S. A., Finlay, G. J., Baguley, B. C., and Askarian-Amiri, M. E. (2016). Signaling pathways in melanogenesis. *Int. J. Mol. Sci.* 17(7): 1144.

Del Marmol, V., and Beermann, F. (1996). Tyrosinase and related proteins in mammalian pigmentation. *FEBS Lett.* 381(3): 165-168.

Franco, A. C., Aveleira, C., and Cavadas, C. (2022). Skin senescence: Mechanisms and impact on whole-body aging. *Trends. Mol. Med.* 28(2): 97-109.

Hearing, V. J., and Tsukamoto, K. (1991). Enzymatic control of pigmentation in mammals. *FASEB J.* 5(14): 2902-2909.

Hemesath, T. J., Price, E. R., Takemoto, C., Badalian, T., and Fisher, D. E. (1998). MAP kinase links the transcription factor microphthalmia to c-Kit signalling in melanocytes. *Nature* 391(6664): 298-301.

Huang, Y.-C., Liu, K.-C., Chiou, Y.-L., Yang, C.-H., Chen, T.-H., Li, T.-T., and Liu, L.-L. (2013). Fenofibrate suppresses melanogenesis in B16-F10 melanoma cells via activation of the p38 mitogen-activated protein kinase pathway. *Chem. Biol. Interact.* 205(3): 157-164.

Kim, A. J., Park, J. E., Cho, Y. H., Lim, D. S., and Lee, J. S. (2021). Effect of 7-methylsulfinylheptyl isothiocyanate on the inhibition of melanogenesis in B16-F1 Cells. *Life* 11(2): 162.

Lee, K. W., Kim, M., Lee, S. H., and Kim, K. D. (2022). The function of autophagy as a regulator of melanin homeostasis. *Cells* 11(13): 2085.

Lei, Y., and Klionsky, D. J. (2021). The emerging roles of autophagy in human diseases. *Biomedicines* 9(11): 1651.

Lin, V. C., Ding, H. Y., Tsai, P. C., Wu, J. Y., Lu, Y. H., and Chang, T.S. (2011). *In vitro* and *in vivo* melanogenesis inhibition by biochanin a from *trifolium pratense*. *Biosci. Biotechnol. Biochem.* 75(5): 914-918.

Liu, D., Meng, X., Wu, D., Qiu, Z., and Luo, H. (2019). A natural isoquinoline alkaloid with antitumor activity: Studies of the biological activities of berberine. *Front. Pharmacol.* 10(2): 9.

Manandhar, B., Wagle, A., Seong, S. H., Paudel, P., Kim, H.-R., Jung, H. A., and Choi, J. S. (2019). Phlorotannins with potential anti-tyrosinase and antioxidant activity isolated from the marine seaweed e*cklonia stolonifera*. *Antioxidants* 8(8): 240.

Moreiras, H., Seabra, M. C., and Barral, D. C. (2021). Melanin transfer in the epidermis: The pursuit of skin pigmentation control mechanisms. *Int. J. Mol. Sci.* 22(9): 4466.

Murase, D., Hachiya, A., Takano, K., Hicks, R., Visscher, M. O., Kitahara, T., Hase, T., Takema, Y., and Yoshimori, T. (2013). Autophagy has a significant role in determining skin color by regulating melanosome degradation in keratinocytes. *J. Invest. Dermatol.* 133(10): 2416-2424.

Sato, K., Takahashi, H., Iraha, R., and Toriyama, M. (2008). Down-regulation of tyrosinase expression by acetylsalicylic acid in murine B16 melanoma. *Biol. Pharm. Bull.* 31(1): 33-37.

Song, Y. C., Lee, Y., Kim, H. M., Hyun, M. Y., Lim, Y. Y., Song, K. Y., and Kim, B. J. (2015). Berberine regulates melanin synthesis by activating PI3K/AKT, ERK and GSK3β in B16F10 melanoma cells. *Int. J. Mol. Med.* 35(4): 1011-1016.

Sridharan, S., Jain, K., and Basu, A. (2011). Regulation of autophagy by kinases. *Cancers* 3(2): 2630-2654.

Wang, N., Feng, Y., Zhu, M., Tsang, C. M., Man, K., Tong, Y., and Tsao, S. W. (2010). Berberine induces autophagic cell death and mitochondrial apoptosis in liver cancer cells: the cellular mechanism. *J. Cell. Biochem.* 111(6): 1426-1436.

Wellbrock, C., and Arozarena, I. (2015). Microphthalmia-associated transcription

factor in melanoma development and MAP-kinase pathway targeted therapy. *Pigment Cell Melanoma Res.* 28(4): 390-406.

Zhang, H., Shan, Y., Wu, Y., Xu, C., Yu, X., Zhao, J., Yan, J., and Shang, W. (2017). Berberine suppresses LPS-induced inflammation through modulating Sirt1/NF-κB signaling pathway in RAW264. 7 cells. *Int. Immunopharmacol.* 52(11): 93-100.

Zhuang, Y., and Lyga, J. (2014). Inflammaging in skin and other tissues-the roles of complement system and macrophage. *Inflamm. Allergy Drug Targets.* 13(3): 153-161.