2023년 2월

박사학위 논문

Effects of 7-methylsulfonylheptyl isothiocyanate on skin whitening, anti-inflammation and autophagy activation

조선대학교 대학원

글로벌바이오융합학과

조 영 희

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피부미백, 항염증 및 **autophagy** 활성화에 미치는
7-methylsulfonylheptyl isothiocyanate의 영향

2023년 2월 24일

조선대학교 대학원

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지도교수 이 정 섭

이 논문을 이학 박사학위 신청 논문으로 제출함.

2022년 10월

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조영희의 박사학위논문을 인준함.

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ABSTRACT

Effects of 7-methylsulfonylheptyl isothiocyanate on skin whitening, anti-inflammation and autophagy activation

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Skin is influenced by extirinsic factors. These factors work synergistically to cause skin aging characterized by melanin hyperpigmentation, wrinkles, loss of skin elasticity, and laxity. Skin maintains cellular homeostasis by activating autophagy system that decomposes intracellular waste products and foreign invading substances. It is also known that the regulation of autophagy can affect inflammatory diseases and aging. Meanwhile, the NLRP3 inflammasome acts as an important role in regulating innate immunity. The compound 7-methylsulfonylhepthyl isothiocynate (7-MSI) routinely used in this study is one of phytochemicals that are abundant in cruciferous plants and is known to show anti-oxidant, -inflammatory, and -cancer effects. However, its effects on skin-lightening, skin aging, and inflammation have not yet been elucidated in detail. Therefore, this study was performed to investigate various effects of 7-methylsulfonylhepthyl isothiocynate on skin-lightening, anti-sikn aging, and anti-inflammation in terms of melanin synthesis, inflammatory cytokine production, inflammasome formation, and autophagy activation. When the melanoma cells (called B16-F1) co-stimulated with 10 nM of α-MSH were treated with 7-methylsulfonylhepthyl isothiocynate (0.5 μg/ml), the rate of melanin synthesis was decreased to roughly 63%, by comparison to that of α-MSH

only group. Western blottings also showed that 7-methylsulfonylhepthyl isothiocynate could reduce the production of melanogenesis-related proteins, including CREB, MITF, TRP1, and tyrosinase. These results propose that 7-methylsulfonylhepthyl isothiocynate can decrease the production of melanin pigment by inhibiting the biochemical pathway of melanogenesis in melanoma cells. On the other hands, the inhibitory effects of 7-methylsulfonylhepthyl isothiocynate on the production of pro-inflammatory cytokines were investigated in macrophage cell. The results of ELSIA and RT-PCR showed that the production of TNF-α as well as the transcriptional levels of pro-inflammatory cytokines were reduced in the cells treated with LPS and 7-MSI for various time periods. In addition, Western blottings (using anti-IκBα and -p-IκBα antibodies) and immunofluorescence microscopy (with anti-NF-κB antibody) showed that 7-MSI had an inhibitory effect on the production of the pro-inflammatory cytokines by the suppression of NF-κB activation in macrophages. In this study, the effect of 7-methylsulfonylhepthyl isothiocynate on the formation of inflammasome was also examined in another macrophage cell line called RAW-ASC cell. When the cells were treated with lipopolysaccharide (0.01 μg/ml), followed by ATP (1 mM) and 7-MSI (2 μg/ml), the expression levels of the components of inflammasome such as NLRP3, ASC, and caspase-1 were all decreased as determined by Western blottings, accompanied with an approximately 80% reduction in IL-1β production as analyzed by ELISA. These results propose that 7-methylsulfonylhepthyl isothiocynate can suppress the formation of inflammasome in immune cells. In this study, the effect of 7-methylsulfonylhepthyl isothiocynate on the activation of autophagy though MAPK pathway was also investigated. Western blottings showed that 7-MSI could increase the expression of MAPK-associated proteins. In addition, the compound could decrease the expression of mTOR, whereas those of typical autophagic proteins increased, together with the increased formation of

autophagosomes as shown by immunofluorescence microscopy. All these results produced that 7-methylsulfonylhepthyl isothiocynate can activate the autophagy system through by the activation of MAPK signaling. In this study, a relationship between the autophagy system and the inflammatory response was also confirmed in melanoma (B16-F1) and macrophage (RAW-ASC) cells that were transfected with siRNAs against Atg5 and Beclin-1. In the B16-F1 cells transfected with Atg5 siRNA, the inhibitory effect of 7-MSI on melanogenesis was reduced. In RAW-ASC cells transfected with Beclin-1 siRNA, the rate of TNF-α production was increased by approximately 5% and the expression levels of the components of inflammasome were also increased. These results propose that 7-methylsulfonylhepthyl isothiocynate can reduce the melanogenesis by activating cellular autophagic system as well as suppress an inflammatory response by inhibiting inflammasome formation. Taken together, the results suggest that 7-MSI can exhibit various effects, such as skin whitening, anti-skin aging, and anti-inflammation through by 1) inhibition of melanin synthesis; 2) inhibition of inflammation on account of the reduction of expression of pro-inflammatory cytokines and the formation of inflammasomes; and 3) activation of the autophagy system through MAPK signaling. In conclusion, all results produced by this study vindivate that 7-MSI has a potential to be developed as a cosmetic material for multifunctional skin anti-aging and skin disease treatment.

1. INTRODUCTION

It has been known that skin aging is caused by intrinsic and extrinsic aging pathways. Intrinsic aging affects the skin of the entire body by genetic factors and hormonal changes. Extrinsic aging is influenced smoking and extrinsic factors. These factors induce skin aging that is associated with hyperpigmentation, inflammation, wrinkles, skin cancer, and increase susceptibility to skin infection (Pilkington *et al.,* 2021) (Fig. 1). Aged skin is identified by skin atrophy resulted from the decreased proliferative ability of skin cells and a degradation of extracellular matrix proteins (Franco *et al.,* 2022).

Melanin is an important factor in determining human skin color and prevents the skin from being damaged by UV-light (Lim *et al.,* 2018). When the skin is damaged by UV-light, keratinocytes produce α-MSH. The α-MSH activate adenylate cyclase by binding to the MC1R in melanocytes. This process amplifies interacellular cAMP signaling and activates PKA and CREB proteins (Seo *et al.,* 2019). The actived CREB increases MITF expression leading to induce the expression of enzymes, such as TRP1/2, DCT, and tyrosinase, which are involved in melanin synthesis (Seo *et al.,* 2019) (Fig. 2). The level of melanin in the skin is determined at the genetic level, however, its amount can also be influenced by excessive exposure to UV radiation, hormones, and age. However, excessive synthesis of melanin causes hyperpigmentation (Ha *et al.,* 2005). Hyperpigmentation of the skin is classified according to the site of deposition. Hyperpigmentation in the epidermis responds best to topical treatment. Pigmented areas appear as distinct freckles with dark brown edges. Hyperpigmentation in the dermis is more difficult to treat because the pigment is distributed in deep structures and appears as irregularly shaped melasma of light brown color with visually

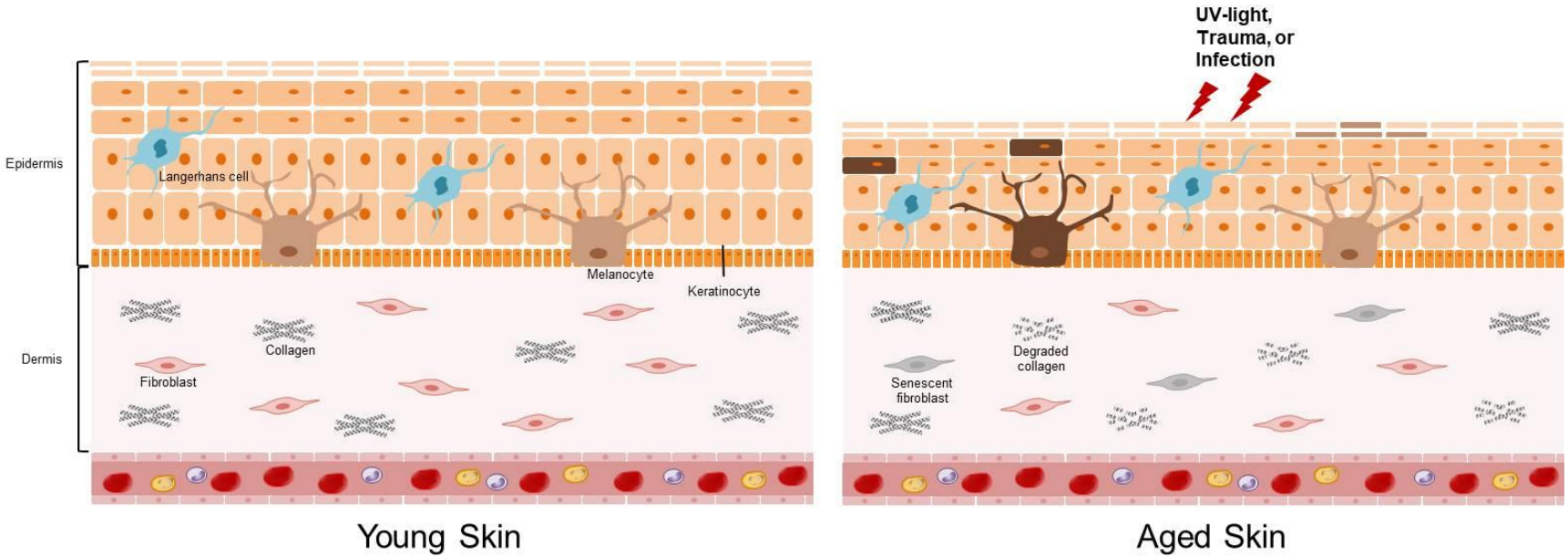


Fig. 1. Schematic representation of skin aging. Aged skin shows an atrophy resulted from the decreased proliferative ability of skin cells and the lowered level of extracellular matrix proteins (Franco *et al.,* 2022).

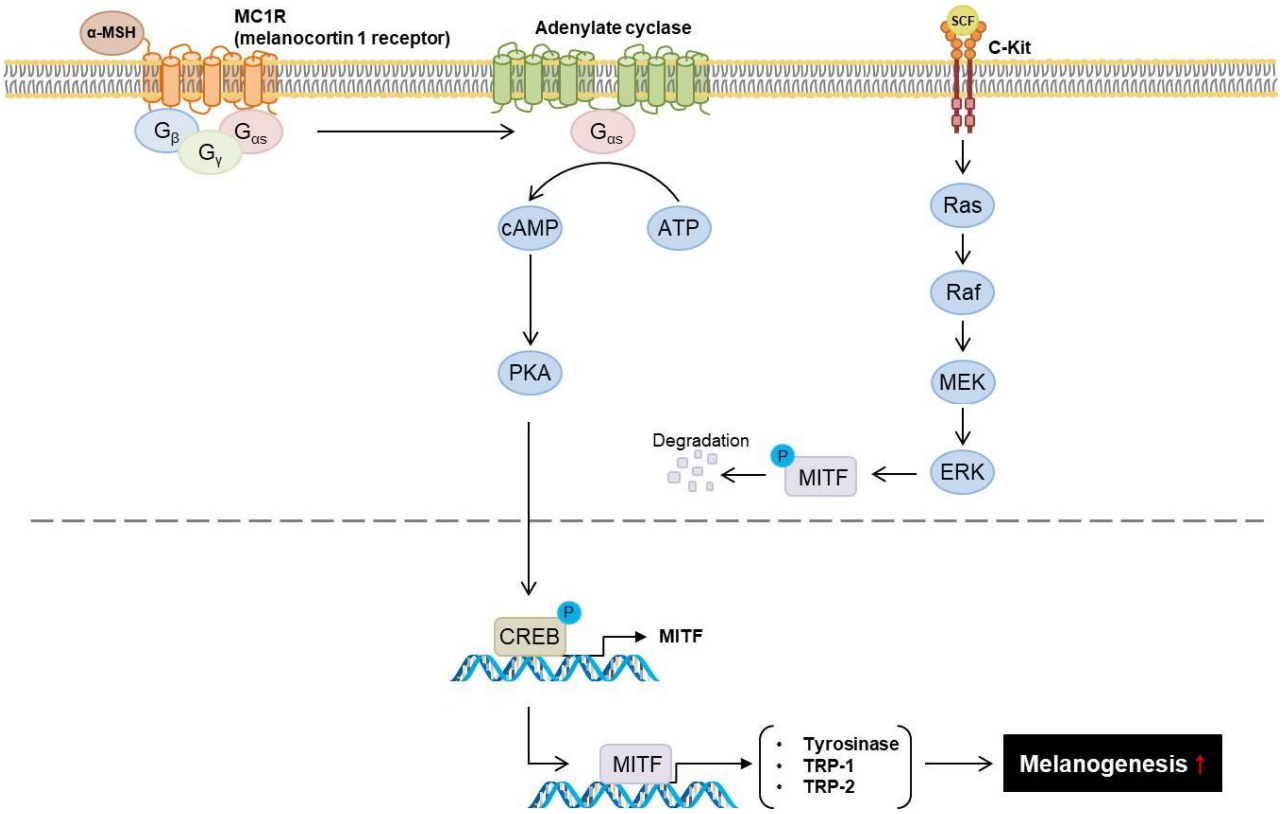


Fig. 2. Melanogenesis in melanocytes. The hormone α-MSH activates adenylate cyclase by binding to the MC1R receptor in melanocytes. This process amplifies interacellular cAMP signaling and activates PKA and CREB proteins (Seo *et al.,* 2019). The activated CREB increases MITF expression, leading to induce the expression of enzymes, which are involved in melanin synthesis (Seo *et al.,* 2019).

indistinct boundaries. When hyperpigmentation is located in the epidermis and dermis, it appears in the form of aged spots. In addition, post-inflammatory hyperpigmentation, an acquired hypermelanosis that occurs after skin inflammation or abrasion in the dermis, releasing large amounts of melanin. The melanin pigments released into the dermis are phagocytosed by macrophages called melanophages, resulting in blue-grayed area. (Fig. 3). It is known that a large amount of keratinocytes exist in the epidermis of the skin, and many immune cells such as dermal dendritic (DC) and mast cells are there in the dermis.

The skin tissue resident cells initiate an immune response as a defense mechanism against damage and infection and recruit neutrophils to the dermis. The neutrophils recruited lead to M1 macrophages to produce pro-inflammatory cytokines involved in promoting inflammatory response, in which M2 macrophages release anti-inflammatory cytokines to counterbalance the response (Jiang *et al.,* 2022) (Fig. 4). As skin aging increases, the pro-inflammatory response of skin cells increases, which can promote an inflammatory skin environment. The nuclear factor kappa-light-chain-enhancer of activated B cells is a nuclear transcription factor concerned in the cellular response to pro-inflammatory stimuli. It exists in the cytosol as an inactive state by binding to IκB but when PAMPs such as LPS are detected by TLR4, the IκB is phosphorylated and released from the complex, making an activated nuclear factor kappa-light-chain-enhancer of activated B cells. The activated nuclear factor kappa-light-chain-enhancer of activated B cells then translocates into the nucleus to induce the expression of pro-inflammatory cytokines (Kuo *et al.,* 2015) (Fig. 5).

The inflammasome is a cytoplasmic multiportein oligomer of the immune system responsible for activating the inflammatory response. Poorly controlled inflammasome activity is known to be associated with serious inflammatory diseases. NLRP3 is known to be important in constructing a multiprotein

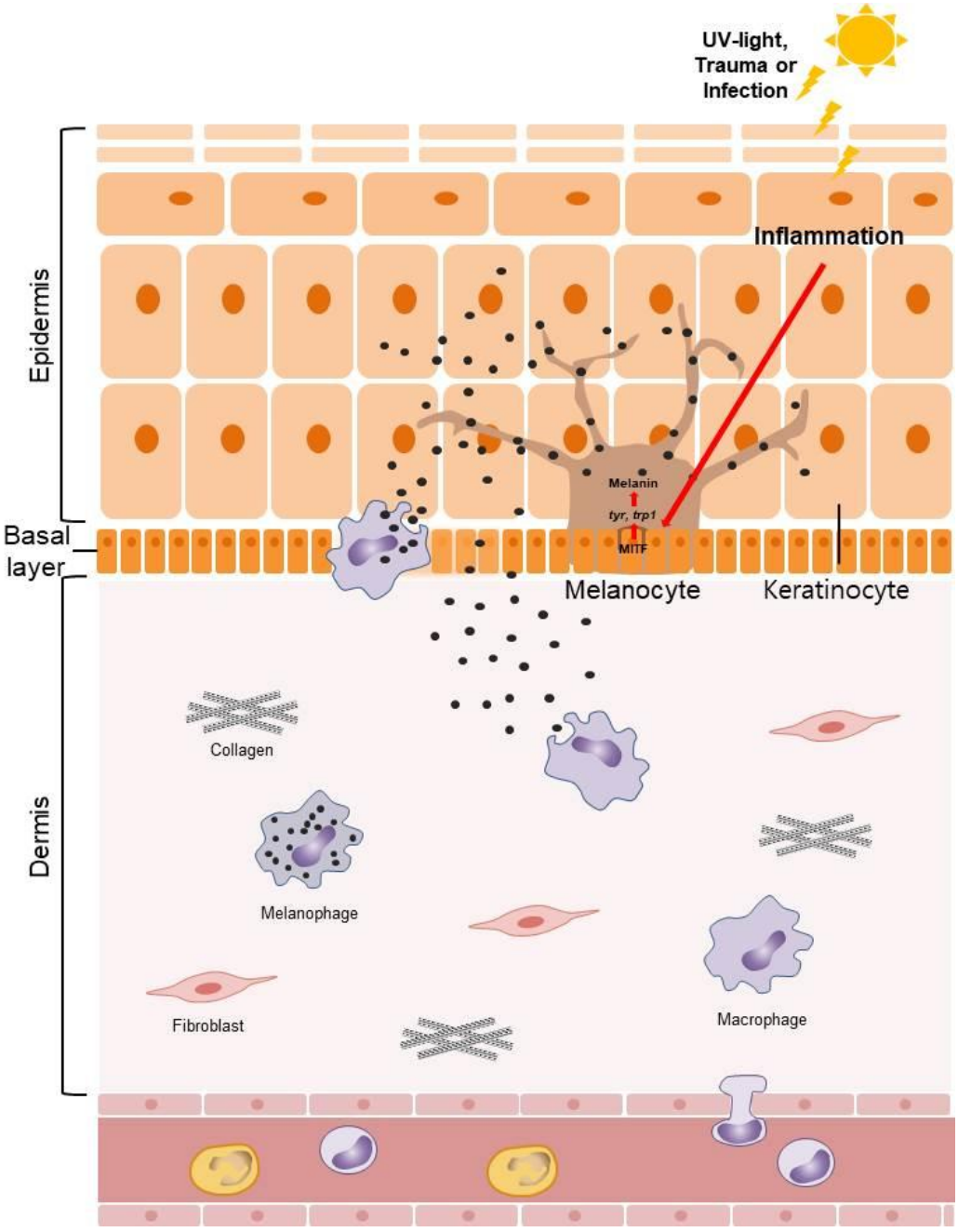


Fig. 3. Skin pigmentation. The melanin pigments released into the dermis are phagocytosed by melanophages that are a sort of macrophages resulting in blue-grayed area in skin.

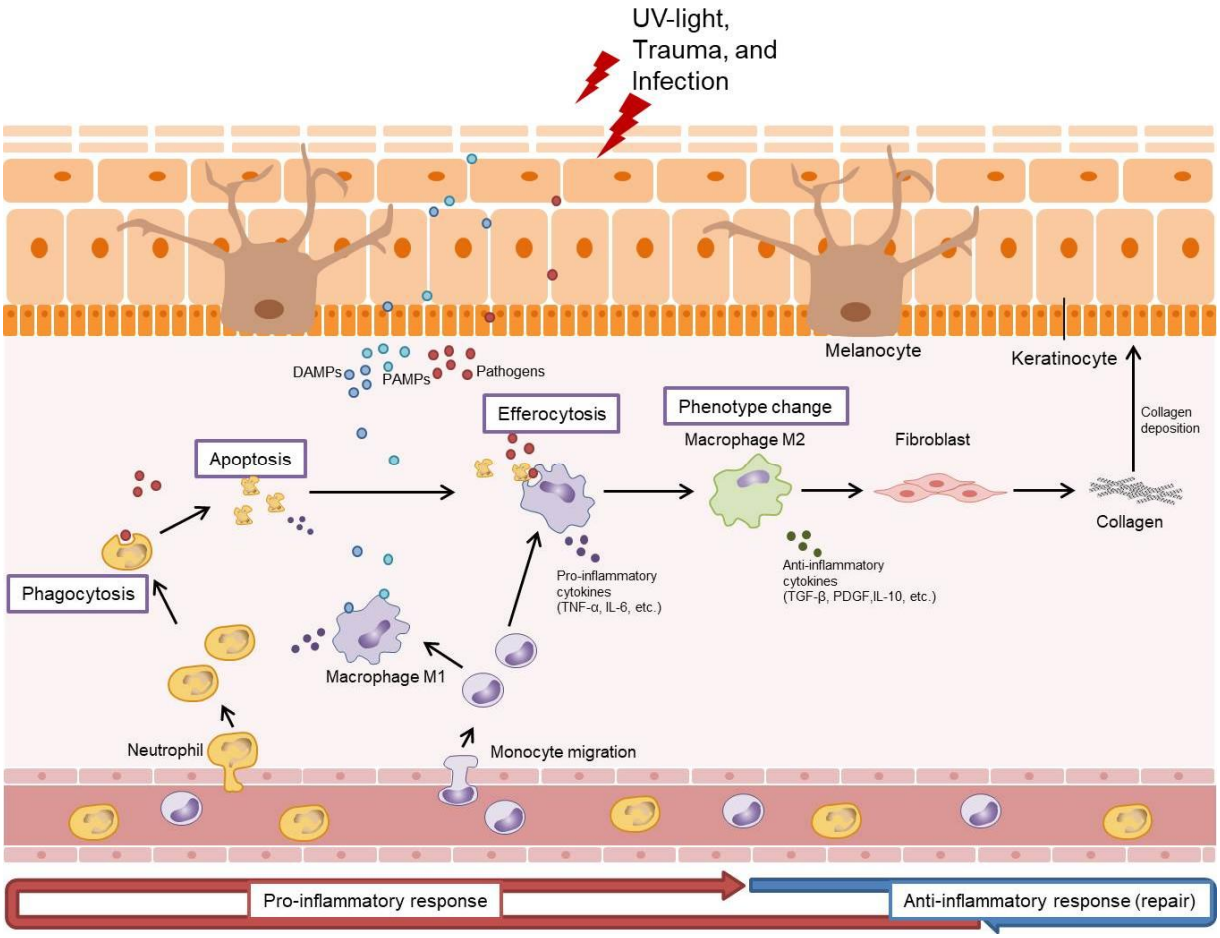


Fig. 4. Inflammatory response in skin. Skin tissue resident cells initiate an immune response as a defense mechanism against damage and infection by recruiting neutrophils to the dermis. The neutrophils recruited lead to M1 macrophages to produce pro-inflammatory cytokines involved in promoting inflammatory response, in which M2 macrophages release anti-inflammatory cytokines to counterbalance the response (Jiang *et al.,* 2022).

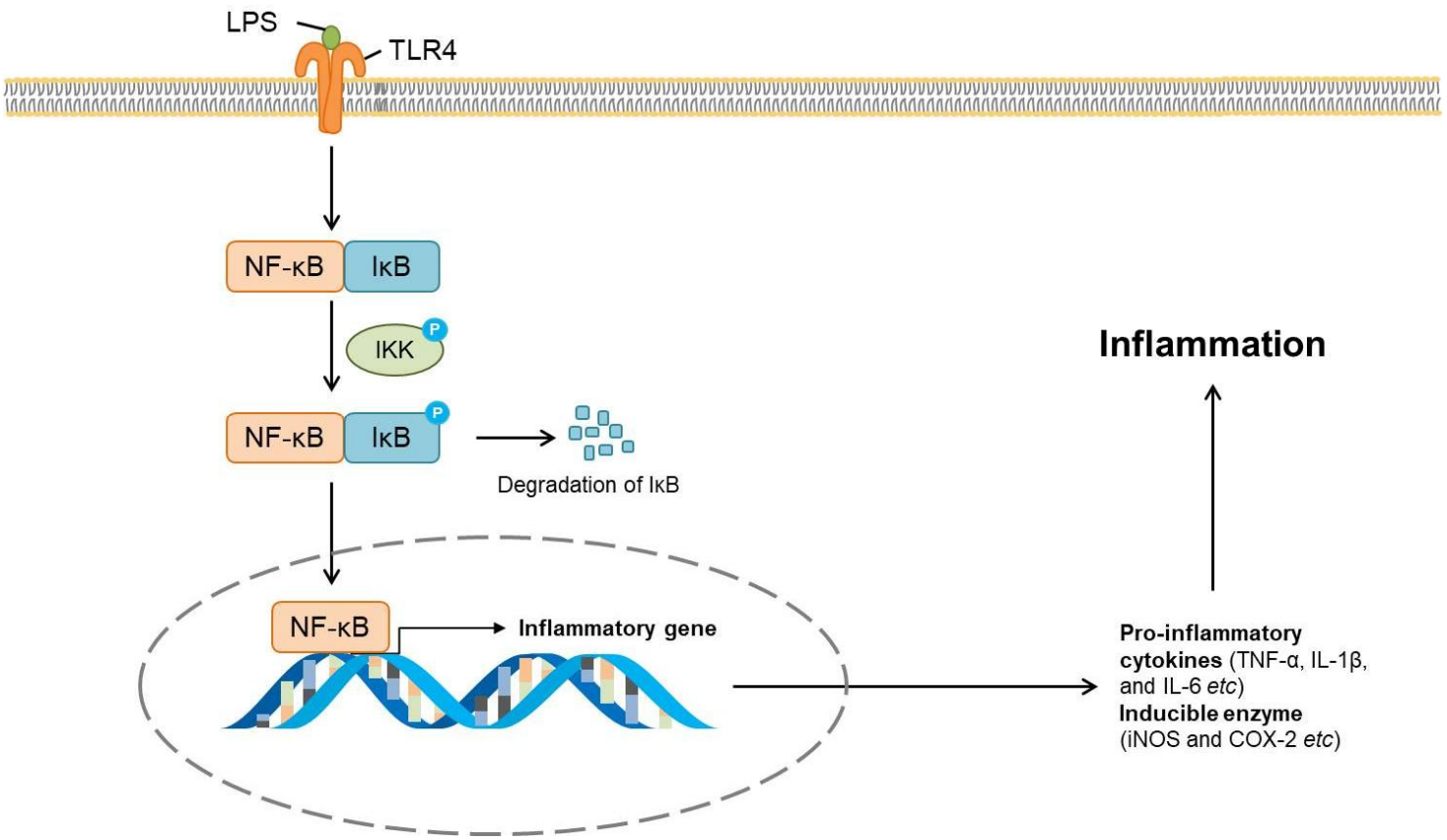


Fig. 5. NF-κB signaling pathway in inflammatory response. NF-κB exists in the cytosol as an inactive state by binding to IκB, but when LPS are detected by TLR4, the IκB is phosphorylated and released from the complex, making an activated nuclear factor kappa-light-chain-enhancer of activated B cells. The activated nuclear factor kappa-light-chain-enhancer of activated B cells then translocates into the nucleus to induce the expression of pro-inflammatory cytokines and pro-inflammatory enzymes (Kuo *et al.,* 2015).

complex of inflammatory regulators called inflammasomes in cells, but the signaling pathway that induces its activity is not well known. The NLRP3 inflammasome acts as a very important regulator of innate immunity, and studies on the including signaling pathway are insufficient (Deets and Vance, 2021). The NLRP3 inflammasome consists of the adapter ASC, the NLR protein NLRP3, and pro-caspase-1. The NLRP3 inflammasome activation process requires two steps known as priming and activating signals. During the priming step, TLR4 binds NF-κB activation stimulants such as LPS induce the expression of inflammasome constituent proteins (Fig. 6). After priming, the second (activating) signal step forms and activates the inflammasome complex by mitochondrial ROS, ATP, and potassium efflux. In the inflammasome generated by second signal, caspase-1 is activated through autoproteolytic maturation, resulting in the release of interleukin-1β and interleukin-18.

Autophagy is a catabolic procedure and allows the orderly degradation and recycling of organelles. Therefore, autophagy helps cell survival under stress conditions such as organelles damage and oxidative stress by supplying cellular energy sources. Nevertheless, the excessive activation of autophagy is also associated with cell death processes and various diseases (Kim *et al*., 2013; Moore 2015). In autophagy, mTORC1 acts as a regulator. Under nutrient-rich conditions, mTORC1 binds the Atg1-Atg13-Atg17-Atg31-Atg29 complex, but not under nutrient-poor conditions. When mTORC1 binds, it phosphorylates ULK1/2 and Atg13 to inactivate them and suppress autophagy. However, when cells are treated with rapamycin or lack of nutrients, mTORC1 is dissociated from the induction complex and dephosphorylated, and the activated ULK1/2 complex moves to the autophagosome nucleation center and proceeds with autophagy (Fujita *et al.,* 2008). The Atg complex and LC3-II bind to the phagophore membrane and the autophagosome is formed (Zhang *et al.,* 2008). Autophagosomes are fused with lysosomes, in which cargos are degraded by hydrolases (Fig. 7). When the cargo is broken down, the product is released

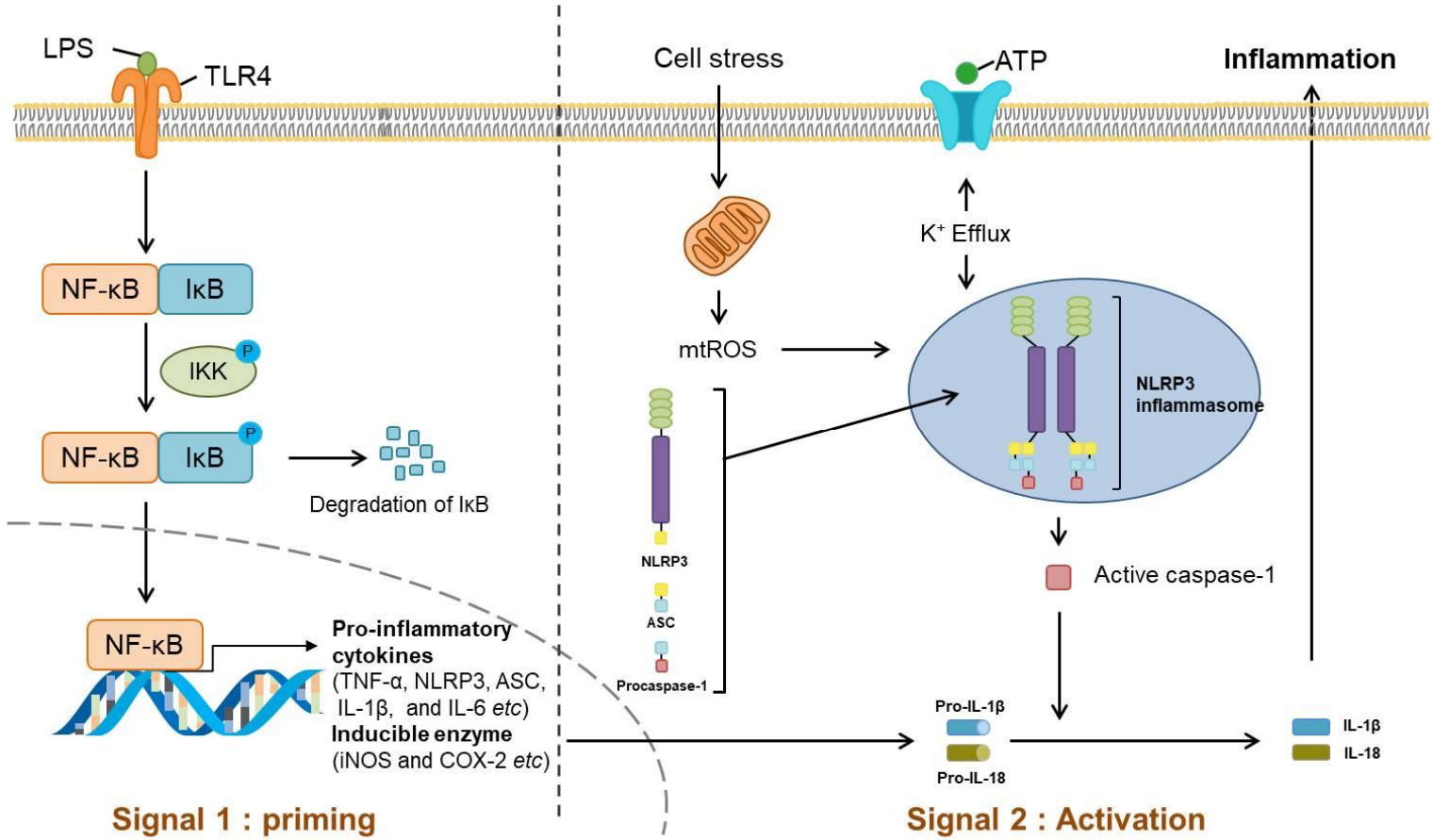


Fig. 6. NLRP3 inflammasome signaling pathway. The NLRP3 inflammasome consists of the adapter ASC, the NLR protein NLRP3, and pro-caspase-1. Two steps are needed to prime the NLRP3 inflammasome. In the inflammasome generated by this second signal, caspase-1 is activated through autoproteolytic maturation, resulting in the secretion of interleukin-1β and interleukin-18 (Deets and Vance, 2021).

into the cytoplasm for reuse in the cell (Karakas *et al.,* 2014). Crosstalk between autophagy, immune, and non-immune cells is required to effectively relieve skin inflammation caused by pathogens (Sil *et al.,* 2018). In the skin, non-immune cells include keratinocyte and melanocyte, and immune cells comprise neutrophil, macrophage, Langerhans cell, and dermal dendritic cell (DC) *etc* (Fig. 8). In melanocytes, autophagy is activated through Atg5 as a mediator. Atg5 deficiency in melanocytes causes premature aging, reactive oxygen species (ROS) accumulation, and melanogenesis (Sil *et al.,* 2018). In addition, macrophages activate autophagy through Beclin-1 as a mediator. In macrophages, Beclin-1 deficiency induces pigmentation and inflammatory responses and inhibits skin repair. It is known that the skin maintains cell homeostasis by activating the autophagy system, decomposes waste or foreign invader in cells, and affects inflammatory disease and aging mechanisms when there is a problem in the autophagy regulation mechanism.

The compound 7-methylsulfonyl hepthyl isothiocynate (7-MSI) used by this study is a synthetic compound related to sulforaphane, which is abundantly in the shoots of cruciferous vegetables such as broccoli and cauliflower. Although a few studies have shown that the compound seems to have an ability to defense against pathogens and exhibit an anti-inflammatory effect (Fig. 9), there was no study on its effects on skin whitening and inflammation in detail. Therefore, this study was performed to reveal the followings: 1) the inhibitory effect of 7-MSI on the production of melanin pigments in murine melanoma cell; 2) the inhibitory effect of 7-MSI on inflammatory response and inflammasome formation in murine macrophage cell; and 3) the effect of 7-methylsulfonyl hepthyl isothiocynate on skin innate immune response and autophagy activation in melanoma and macrophage cell.

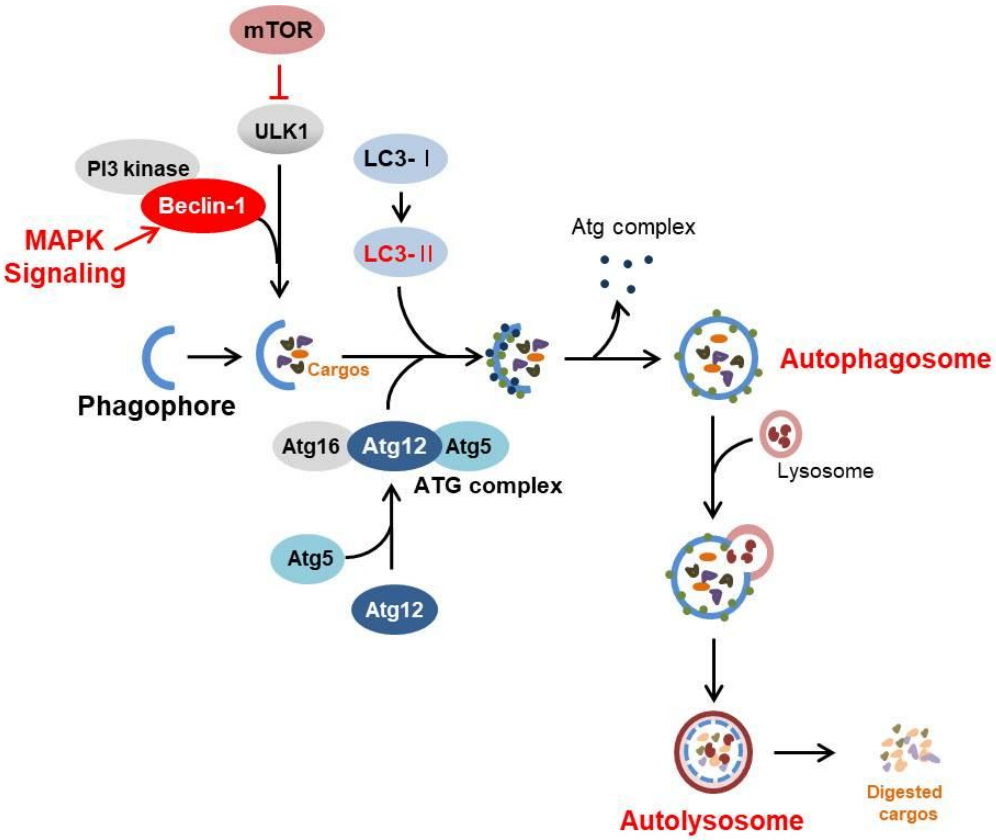
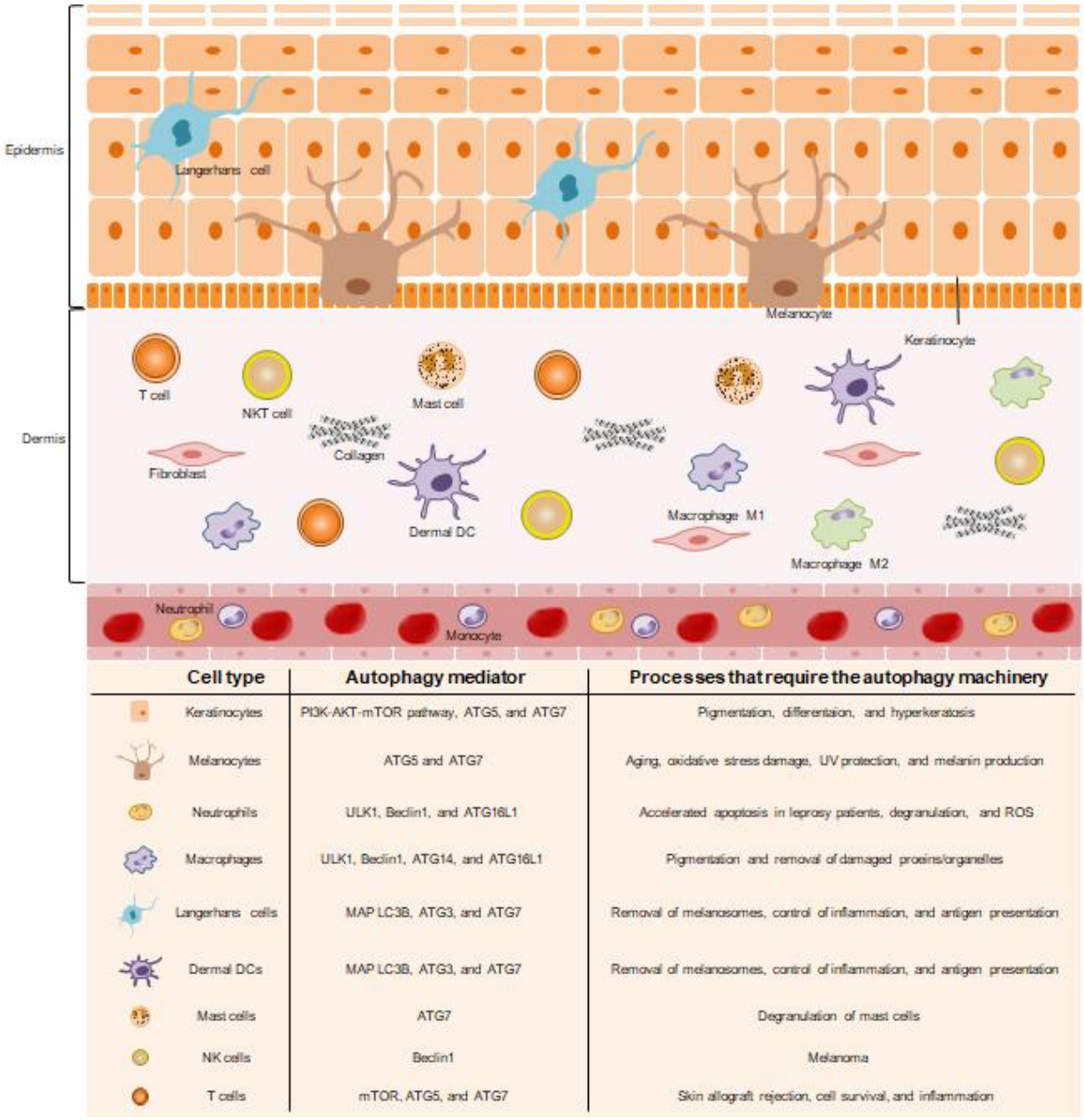


Fig. 7. Autophagy signaling pathway. The mTOR is a major negative controller for autophagy. Beclin-1 through MAPKs activation induces autophagy activation. After that, the Atg complex and LC3-II bind to the phagophore membrane and the autophagosome is formed. Autophagosomes are fused with lysosomes, in which cargos are degraded by hydrolases (Zhang *et al.,* 2008).



Cell type	Autophagy mediator	Processes that require the autophagy machinery
Keratinocytes	PI3K-AKT-mTOR pathway, ATG5, and ATG7	Pigmentation, differentiation, and hyperkeratosis
Melanocytes	ATG5 and ATG7	Aging, oxidative stress damage, UV protection, and melanin production
Neutrophils	ULK1, Beclin1, and ATG16L1	Accelerated apoptosis in leprosy patients, degranulation, and ROS
Macrophages	ULK1, Beclin1, ATG14, and ATG16L1	Pigmentation and removal of damaged proeins/organelles
Langerhans cells	MAP LC3B, ATG3, and ATG7	Removal of melanosomes, control of inflammation, and antigen presentation
Dermal DCs	MAP LC3B, ATG3, and ATG7	Removal of melanosomes, control of inflammation, and antigen presentation
Mast cells	ATG7	Degranulation of mast cells
NK cells	Beclin1	Melanoma
T cells	mTOR, ATG5, and ATG7	Skin allograft rejection, cell survival, and inflammation

Fig. 8. The pertinent autophagy components in skin cells. Crosstalk between autophagy, immune, and non-immune cells is required to relieve skin inflammation caused by pathogens (Sil *et al.,* 2018). In the skin, non-immune cells include keratinocyte and melanocyte, and immune cells comprise neutrophil, macrophage, Langerhans cell, and dermal dendritic cell (DC) *etc*.

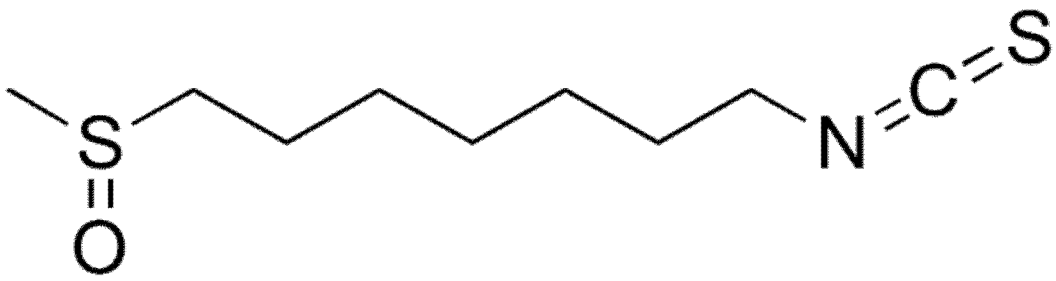


Fig. 9. Physical structure of 7-methylsulfonylheptyl isothiocynate (7-MSI). This phytochemical is abundant in the shoots of cruciferous vegetables, including broccoli and cauliflower (Lim *et al.,* 2017).

2. MATERIALS AND METHODS

2-1. Materials

The compound, 7-methylsulfonyl hepthyl isothiocynate (7-MSI) was obtained from LKT Lab (MN, USA). Dulbecco's modified eagle medium (DMEM; Lonza, Swiss), fetal bovine serum (FBS; ATLAS, CO, USA), penicillin-streptomycin (Sigma-Aldrich, MO, USA), normocin, and blasticidin (InvivoGen, CA, USA) were routinely used for cell culture. ATP was purchased from InvivoGen (CA, USA). Arbutin, ploy-L-lysine, α-MSH, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (MO, USA). Transforming growth factor-β (TGF-β) was purchased from R&D system (MN, USA). Antibodies raised against NF-κB, p65, tyrosinase, and TRP1 were purchased from Santa Cruz Biotechnology (TX, USA). Antibodies raised against GAPDH, LC3, Beclin-1, Atg12, p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2), p38, phospho-p38, MITF, mTOR, phospho-mTOR, IκB-α, phospho-IκB-α, JNK, phospho-JNK, ASC, NLRP3, and Caspase-1 were purchased form Cell signaling (MA, USA).

2-2. Cell culture

Murine melanoma (B16-F1) and macrophage (Raw 264.7) cells were purchased from American type culture collection (ATCC, VA, USA). Another murine ASC-expressing Raw 264.7 cells (so called RAW-ASC) was purchased from InvivoGen (CA, USA). The cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 50 ㎍/ml blasticidin (RAW-ASC cells), and 100 ㎍/ml normocin (RAW-ASC cells) in a 5% CO_2

incubator at 37℃.

2-3. Cell viability assay

Cell viability was evaluated using the CellTiter 96® AQueous Non-Radioactive cell proliferation assay (Promea, WI, USA). B16-F1 cells were cultured at a density of 0.5 × 10^5 cells/well in 96 well plates overnight, and then treated with various concentration of 7-MSI (0, 0.1, 0.5, 1, 2, and 4 μg/ml) or TGF-β (0, 0.00002, 0.0002, 0.002, 0.02, and 0.2 ng/ml) as a positive control for 24 h. Raw 264.7 cells were cultured at a density of 0.5 × 10^5 cells/well in 96 well plates overnight, and then treated with various concentrations of 7-MSI (0, 0.1, 0.5, 1, 3, and 5 μg/ml) or TGF-β (0, 0.00002, 0.0002, 0.002, 0.02, and 0.2 ng/ml) for 24 h. At the end of the incubation, 20 μl of MTS dye solution was added to each well. The cells were further incubated at 37℃ in 5% CO_2 for 4 h. The absorbance was measured at a wavelength of 490 nm using a 96-well platereader.

2-4. 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 10 nM of α-MSH plus various concentrations of 7-MSI (0, 0.05, 0.1, 0.5, 1, and 2 μg/ml) for 72 h at 37℃. The culture supernatant was collected after centrifugation at 16,000 xg for 4 min. The extracellular melanin content was measured by analyzing the absorbance at 490 nm.

2-5. Western blot analysis

B16-F1 cells were seeded at a density of 0.5 × 10^5 cells/well in 6-well plates. After culturing overnight, to induce melanogenesis, the cells were treated with α-MSH (10 nM) and then treated with 7-MSI (1 μg/ml) for 24 h at 37℃ in 5% CO_2. Raw 264.7 and RAW-ASC cells were seeded at a density of 2.0 × 10^5 cells/well in 6-well plates. After culturing overnight, to induce inflammatory response and inflammasome formation. Raw 264.7 cells were treated with LPS (0.01 or 1 μg/ml) and 7-MSI (1 or 2 μg/ml) for 0.5, 1, and 3 h at 37℃ in 5% CO_2. RAW-ASC cells were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h at 37℃ in 5% CO_2. The protein extracts were isolated from the cells using a ProNA CETi lysis buffer (TransLab, Daejeon, Korea). The 50 or 100 μg of proteins were boiled at 100℃ for 3 min and then loaded onto 8%, 10%, 12%, or 15% polyacrylamide gel. For Western blot analysis, protein samples were subjected to SDS-PAGE and then transferred onto a PVDF membrane (Bio-Rad, CA, USA). The membrane was blocked with 5% skim milk in TBS-T (250 mM Tris-HCl, pH 8.0, 1.5 mM NaCl, and 0.1% Tween 20) at room temperature (RT) for 2 h. The membrane was then incubated with specific primary antibodies (1:500 or 1:1000 in blocking buffer) overnight at 4 ℃, and washed six times with TBS-T buffer. The membrane was then incubated with HRP-conjugated secondary antibodies (1:4000 in the blocking buffer) at RT for 2 h. After washing five times with TBS-T buffer, the protein expression levels were determined by analyzing the chemiluminescence captured on the PVDF membrane by EZ-Western Lumi Plus and Lumi Femto (DAEILLAB SERVICE, Seoul, Korea) and exposed on X-ray film (Fuji film, Tokyo, Japan). Western blot data was quantified using the program ImageJ (National Institutes of Health, MD, USA).

2-6. Enzyme-linked immunosorbent assay (ELISA)

Raw 264.7 and RAW-ASC cells were seeded on 24-well plates at a density of 0.25 × 10^5 cells/well and incubated overnight. The cells were treated with 7-MSI (0, 0.1, 1, 2, 3, and 4 μg/ml) in the presence of LPS (1 μg/ml) for 1 h at 37°C in 5% CO_2. The TNF-α protein levels in the culture supernatant were determined using an ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instruction. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, CA, USA). RAW-ASC cells were seeded on 6-well plates at a density of 2 × 10^5 cells/well and incubated overnight. Raw-ASC cells were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h at 37℃ in 5% CO_2. The IL-1β protein levels in the culture supernatant were determined using an ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instruction. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, CA, USA).

2-7. Total RNA purification and cDNA synthesis

Raw 264.7 cells were plated on 12-well plates at a density of 0.1 × 10^5 cells/well and incubated overnight. The cells were treated with 7-MSI (0, 0.5, 1, and 2 μg/ml) in the presence of LPS (1 μg/ml) for 3 h. Raw-ASC cells were seeded on 6-well plates at a density of 2 × 10^5 cells/well and incubated overnight. The cells were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h at 37℃ in 5% CO_2. The cells were lysed and total RNA was isolated by an RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's

instructions. First-strand cDNA was synthesized from 500 μg of RNA with oligo $(dT)_{18}$ primers using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea). Cycling conditions were for 5 min at 25°C, for 1 h at 42°C, and for 15 min at 70°C.

2-8. Reverse transcription-polymerase chain reaction (RT-PCR)

The cDNA products were used as templates for PCR to amplify the target genes. PCR was performed using Top DNA polymerase (Bioneer, Daejeon, Korea). Cycling conditions were as follows; denaturation for 30 sec at 94°C, annealing for 30 sec at 48°C, 55°C, or 60°C, and extension for 30 sec at 72°C for 30 cycles. The sequences of the specific primers used were as follows: IL-1β forward 5′-TGTGAAATGCCACCTTTTGA-3′ and reverse 5′-GTAGCTGCCACAGCTTCTCC-3′, IL-6 forward 5′-AATTTCCTCTGGTCTTC-TGG-3′ and reverse 5′-TAGCCACTC CTTCTGTGACTC-3′, COX-2 forward 5′-CAGCAAATCCTTGCTGTTCC-3′ and reverse 5′-CCATCCTTGAAAAGGC-GCAG-3′, PGEs forward 5′-ATGCCTTCCCCGGGCCTG-3′ and reverse 5′-TCACAGATGGTGGGCCAC-3′, NLRP3 forward 5′-CCTGACCCAAACCCAC-CAGT-3′ and reverse 5′-TTCTTTCGGATGAGGCTGCTTA-3′, ASC forward 5′-TGAGCAGCTGCAAACGACTA-3′ and reverse 5′-ACACTGCCATGCAAAGC-ATC-3′, Caspase-1 forward 5′- ATGAATCACCAACACCAG-3′ and reverse 5′-CTTGACGCATCCTAATCC-3′, and GAPDH forward 5′-TCAGCAA TGCATCC-TGCACCAC-3′ and reverse 5′-TGCCAGTGAGCTTCCCGTTCAG-3′.

2-9. Immunostaining for confocal microscopic analysis

B16-F1 cells were seeded at a density of 0.1×10^5 cells/well and

Raw 264.7 and RAW-ASC cells were seeded at a density of 1.0 × 10^5 cells/well on poly-L-lysine (0.01% solution)-coated glass coverslip in 12-well plates. After culturing for 24 h, the cells were treated with 10 nM α-MSH with 2 ㎍/ml 7-MSI or 10 ng/ml TGF-β for 15 min or 30 min at 37°C in 5% CO_2. Raw 264.7 cells were treated with 7-MSI (1 μg/ml) in the presence of LPS (1 μg/ml) for 1 h at 37°C in 5% CO_2. RAW-ASC cells were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h. The cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at RT. After washing three times with PBS, the cells were permeabilized with 0.1% Triton X-100 for 10 min at RT and then washed with PBS for three times. The cells were then blocked with 1% BSA in PBS for 20 min at RT. After washing three times with PBS, the cells were incubated with a 1:50 dilution of anti-MAP LC3, anti-NF-κB p65, anti-ASC, or anti-Caspase-1 antibody for 1 h at RT. After incubating for 1 h at RT, the cells were washed three times with PBS. The cells were incubated secondary antibody Alexa Fluor 488 goat anti-mouse IgG (diluted 1:200 with PBS; Invitrogen, MA, USA) and goat anti-rabbit IgG-TRITC (diluted 1:200 with PBS; Santa cruz, TX, USA) for 1 h at RT. The cells were washed with PBS for three times, stained with 4'6-diamidino-2-phenylindole (DAPI), and observed using a Zeiss LSM 510 confocal microscope (LePecq, France).

2-10. Caspase-1 activity assay

Caspase-1 activity assay were performed with fluorometric Caspase-1 assay kit (abcam, Cambridge, UK) according to manufacturer's instruction. RAW-ASC cells cultured overnight in 6-well plate at a density of 2 × 10^5 cells/well were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h at 37℃ in 5% CO_2. After

washing with cold PBS, the cells were resuspended in 50 μl of the cell lysis buffer. After incubating on ice for 10 min, the cell lysates were centrifuged at 16,000 xg for 4 min at 4℃ and the resulting supernatants were collected to assay caspase-1 activity. Briefly, 50 μl of the sample collected were mixed with 50 μl of 2X reaction buffer (containing 10 mM DTT) in a 96-well black plate. After adding 50 μM of YVAD-AFC substrate and then incubating at 37℃ for 1 h, the fluorescence intensity was measured at excitation and emission wavelengths of 400 nm and 505 nm, respectively, using a microplate reader (Molecular Devices, CA, USA).

2-11. Transfection of siRNA into B16-F1 and RAW-ASC cells

B16-F1 cells and RAW-ASC were transfected with 400 pmols each of Atg5 and Beclin-1 small interfering RNA (siRNA; Bioneer, Daejeon, Korea) with siRNA transfection reagent and suspended in siRNA transfection medium (serum-free medium) at 37°C in 5% CO_2 for 24 h. The non-transfected control and transfected cells were used as described previously (Rai *et al*., 2013).

2-12. Statistical analysis

All statistical data and graphs were analyzed using SigmaPlot 10.0 (Systat Software Inc., CA, USA). Data were expressed as mean ± standard error of mean (S.E.M) and statistical significance was determined by *t*-test using SigmaPlot software. $p < 0.05$ was considered a statistical significance.

3. RESULTS AND DISCUSSION

3-1. Effects of 7-MSI on skin whitening

3-1-1. Effect of 7-MSI on cell survival in murine melanoma cell line (B16-F1)

To investigate the effect of 7-methylsulfonyl hepthyl isothiocynate on cell survival, MTS assay was performed with melanoma cells after treatments with diverse concentrations of 7-methylsulfonyl hepthyl isothiocynate (0 - 4 μg/ml). As shown in Fig. 10, 7-MSI did not show any cytotoxic effects up to 4 μg/ml, compared to non-treated control. These results suggest that 7-methylsulfonyl hepthyl isothiocynate has no cytotoxic effect on B16-F1 cells.

3-1-2. Inhibitory effect of 7-MSI on melanogenesis in B16-F1 cells

It has been well known that the active CREB increases MITF expression to induce the productions of enzymes, including TRP 1/2, DCT, and tyrosinase, which are involved in melanin synthesis (Seo *et al.,* 2019). In this study, the inhibitory effect of 7-methylsulfonyl hepthyl isothiocynate on the expression levels of melanogenesis-related proteins were examined using Western blottings. As shown in Fig. 11, the production levels of MITF, CREB, p-CREB, TRP-1, and tyrosinase were decreased by 28%, 9%, 27%, 11%, and 45%, respectively, in the cells treated with 1 μg/ml of 7-methylsulfonyl hepthyl isothiocynate in the presence of α-MSH (10 nM), as

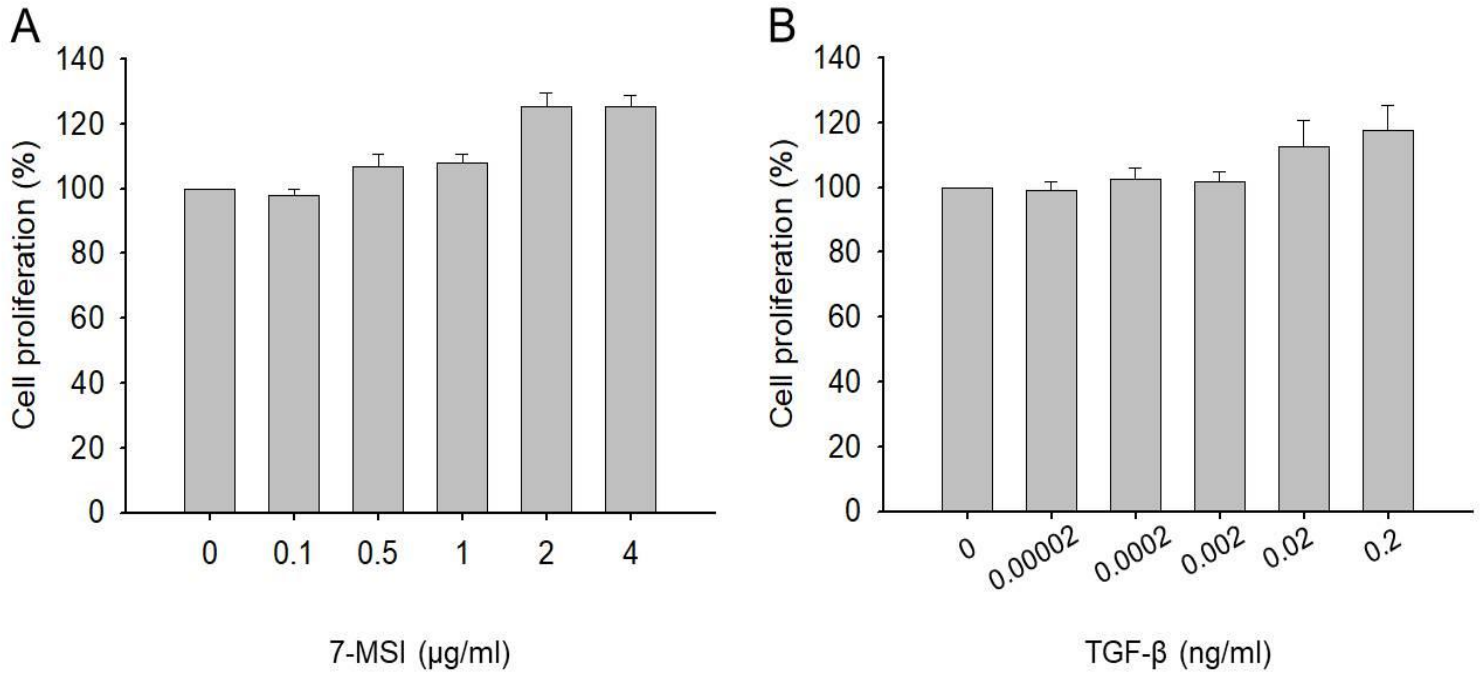


Fig. 10. Effect of 7-MSI on the cell proliferation of B16-F1 cells. Melanoma cells were treated with diverse concentrations of 7-methylsulfonyl hepthyl isothiocynate (0 - 4 μg/ml) (A) and TGF-β (0 - 0.2 ng/ml) (B) and the cell survivals were examined by MTS assay as described in the Materials and Methods.

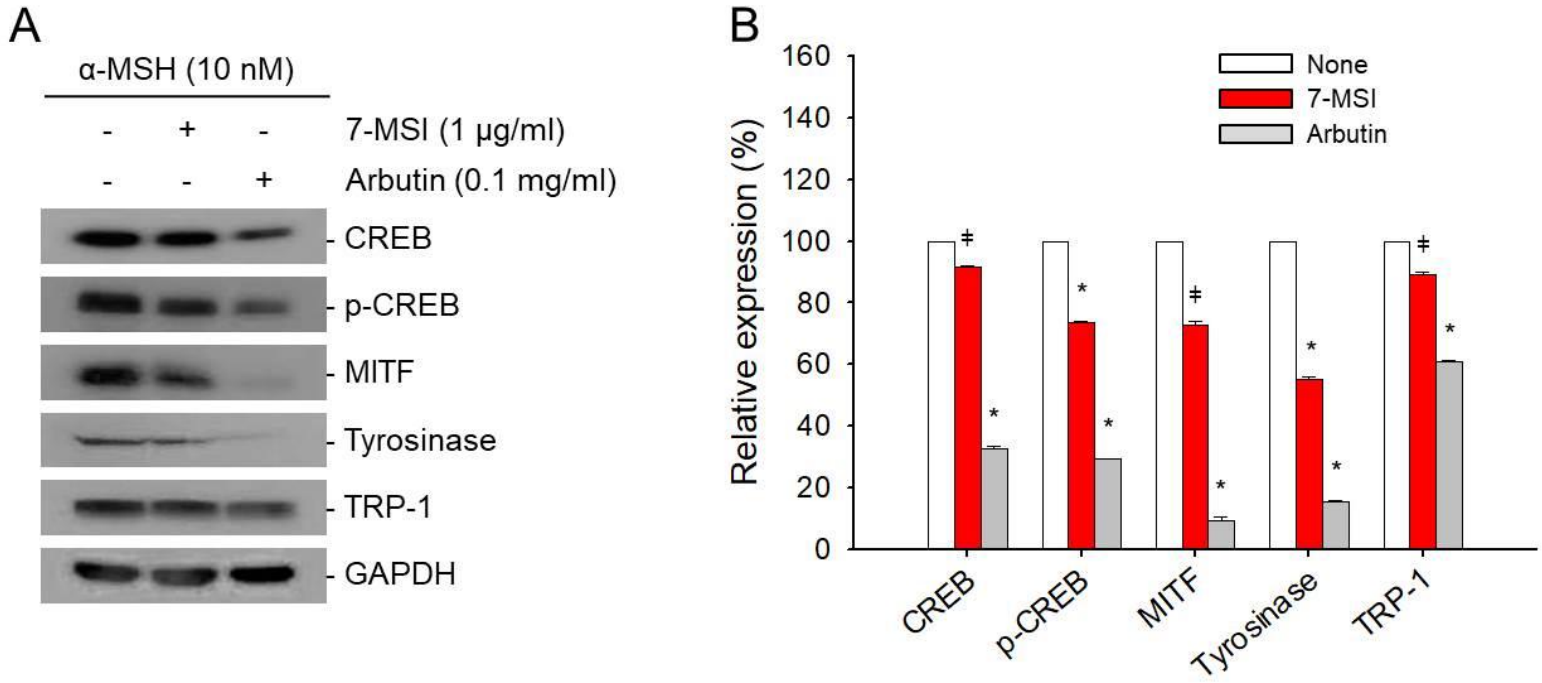


Fig. 11. Effects of 7-MSI on the expression of various melanogenesis-associated proteins in B16-F1 cells. (A) The inhibitory effect of 7-methylsulfonyl hepthyl isothiocynate on the melanogensis-associated protein expressions. B16-F1 cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) or arbutin (0.1 mg/ml), from which proteins were prepared and Western blottings were performed to examine the expressions of MITF, CREB, p-CREB, TRP-1, Tyrosinase, and GAPDH. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

compared to α-MSH only group. These results produced that 7-methylsulfonyl hepthyl isothiocynate can suppress the production of melanogenesis-related proteins.

3-1-3. Inhibitory effects of 7-MSI on melanin synthesis in B16-F1 cells

To investigate the inhibitory effect of 7-methylsulfonyl hepthyl isothiocynate on melanin production, melanoma cells were treated with 7-methylsulfonyl hepthyl isothiocynate (0 - 2 μg/ml) in the presence of α -MSH (10 nM). As shown in Fig. 12, the rate of melanin synthesis could be decreased dose-dependently by the treatment with 7-methylsulfonyl hepthyl isothiocynate, in which 1 μg/ml of 7-methylsulfonyl hepthyl isothiocynate decreased to about 62%, in comparison with that of α-MSH only group. These results produced that 7-methylsulfonyl hepthyl isothiocynate can directly suppress the melanin product in melanoma cells.

3-2. Effect of 7-MSI on inflammatory response

3-2-1. Effect of 7-MSI on cell survival in murine macrophage cell line (Raw 264.7)

To investigate that 7-methylsulfonyl hepthyl isothiocynate can affect on cell survival, MTS assay was performed with macorphage cells after treatments with diverse concentrations of 7-methylsulfonyl hepthyl isothiocynate (0 - 5 μg/ml). As shown in Fig. 13, 7-methylsulfonyl hepthyl isothiocynate did not show any cytotoxic effects up to 5 μg/ml. These results produced that 7-methylsulfonyl hepthyl isothiocynate has no cytotoxic effect

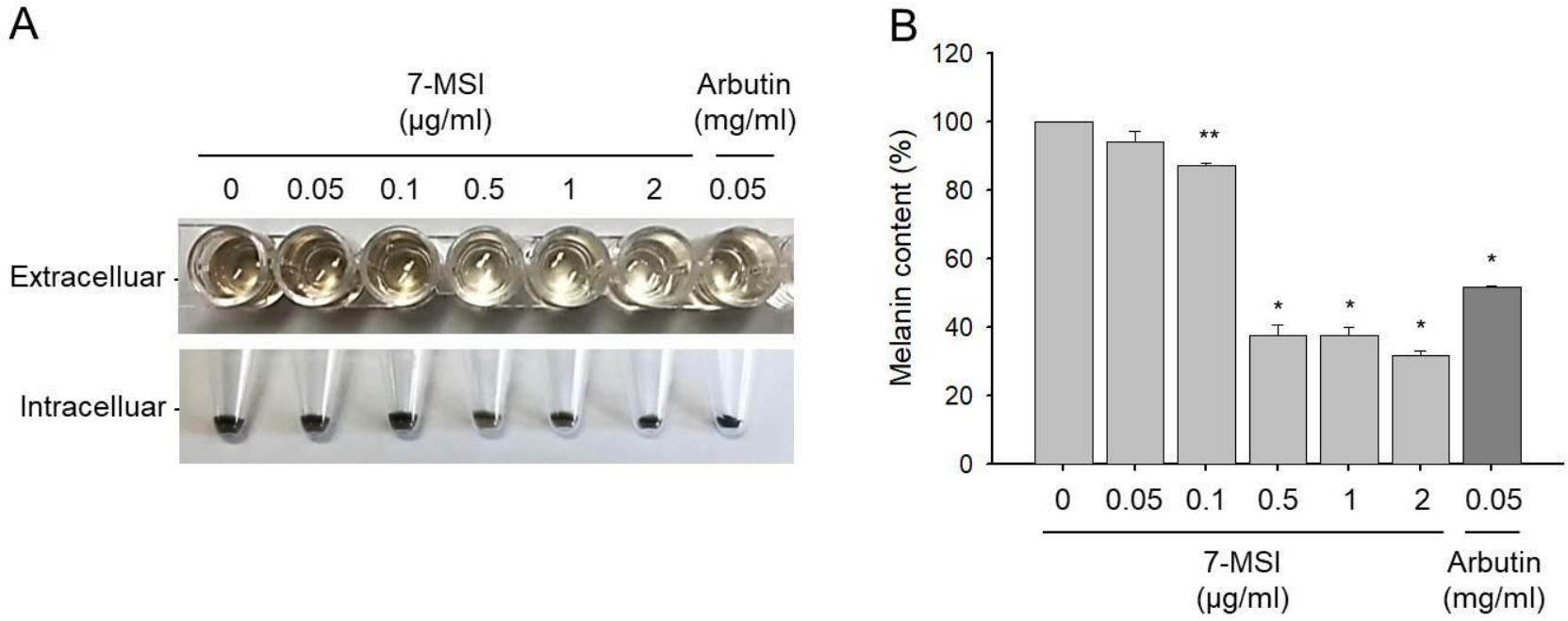


Fig. 12. Inhibition of melanin synthesis by 7-MSI in B1-6F1 cells. (A) B16-F1 cells were treated with diverse density of 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) as indicated and the melanin contents in culture media were examined by measuring A_{490}. (B) Histograms showing the melanin contents.

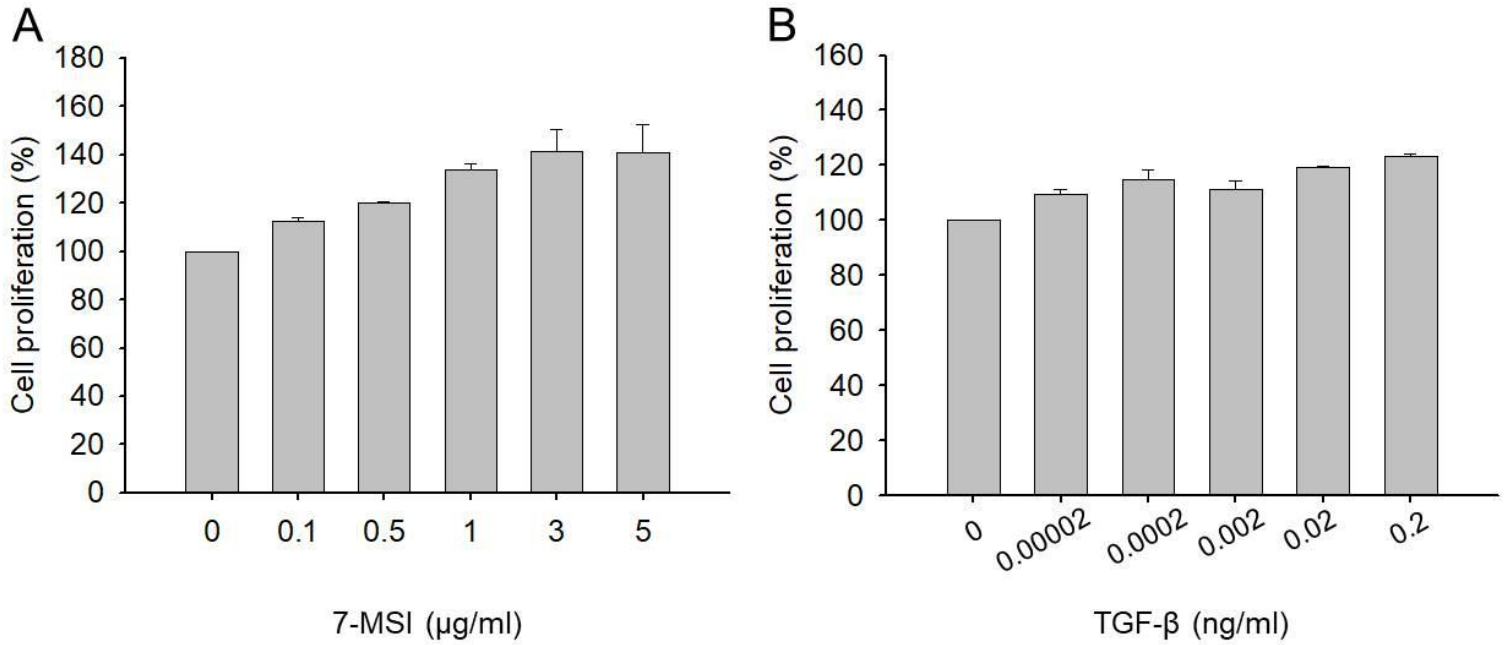


Fig. 13. Effect of 7-MSI on the rate of cell survival of Raw 264.7 cells. Macrophage cells were treated with diverse density of 7-MSI (0 - 5 μg/ml) (A) and TGF-β (0 - 0.2 ng/ml) (B) and the cell survivals were examined by MTS assay as described in Materials and Methods.

on macrophage cells.

3-2-2 Effects of 7-MSI on TNF-α production in Raw 264.7 cells

To examine the effects of 7-MSI on the production of TNF-α in LPS-stimulated macrophage cells, ELISA was performed. As shown in Fig. 14, the production of TNF-α clearly increased to 1,106 pg/ml when macrophage cells were treated with 1 μg/ml of lipopolysaccharide for 1 h. However, the TNF-α level was decreased to 752 pg/ml, when the cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml). These results produced that 7-methylsulfonyl hepthyl isothiocynate has an anti-inflammatory effect by suppressing TNF-α production.

3-2-3 Effects of 7-MSI on the transcription levels of various pro-inflammatory cytokines in Raw 264.7 cells

Once activated, macrophages produce pro-inflammatory cytokines that act as positive regulators for inflammatory reactions. In this study, the expression levels of pro-inflammatory cytokines and regulators were analyzed in macorphage cells using RT-PCR. When the cells were treated 7-methylsulfonyl hepthyl isothiocynate (0 - 2 μg/ml) in the existence of lipopolysaccharide (1 μg/ml), the transcription levels of interleukin-1β, COX-2, interleukin-6, and PGEs significantly were decreased by approximately 562%, 209%, 126%, and 56%, respectively, in comparison with those of the LPS-only control group (Fig. 15). These results produced that 7-methylsulfonyl hepthyl isothiocynate can inhibit LPS-induced inflammatory response though suppressing the expression of pro-inflammatory cytokines and regulators.

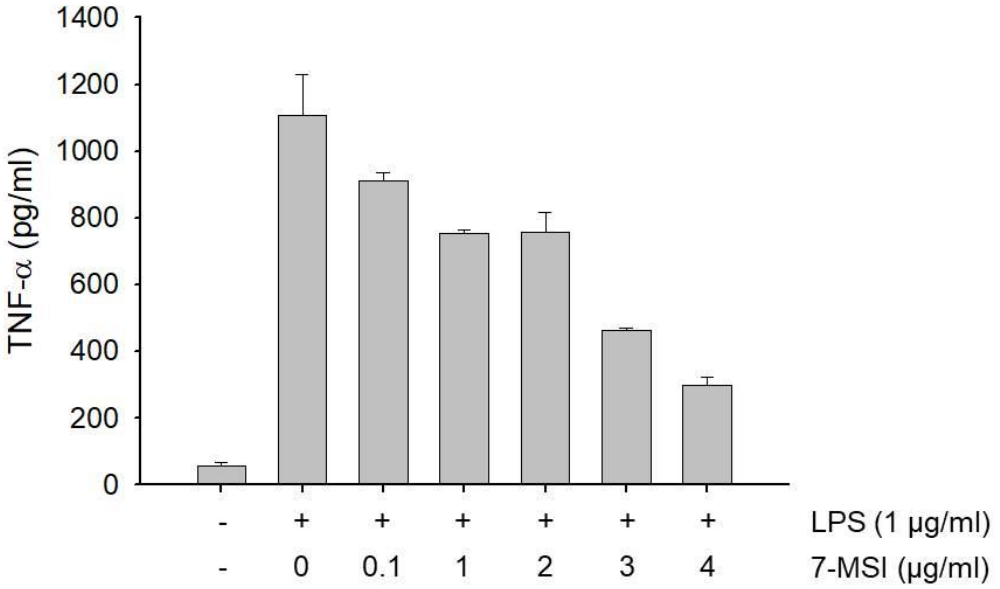


Fig. 14. Effect of 7-MSI on TNF-α production in Raw 264.7 cells. Macrophage cells were treated with 7-MSI (0.1 - 4 μg/ml) in the expression of lipopolysaccharide (1 μg/ml). TNF-α density in the culture supernatants were measured using an ELISA kit specific for TNF-α. The symbols “+” and “-” indicate the addition and the omission of corresponding additives, respectively. The concentration of TNF-α was compared to those from a standard curve.

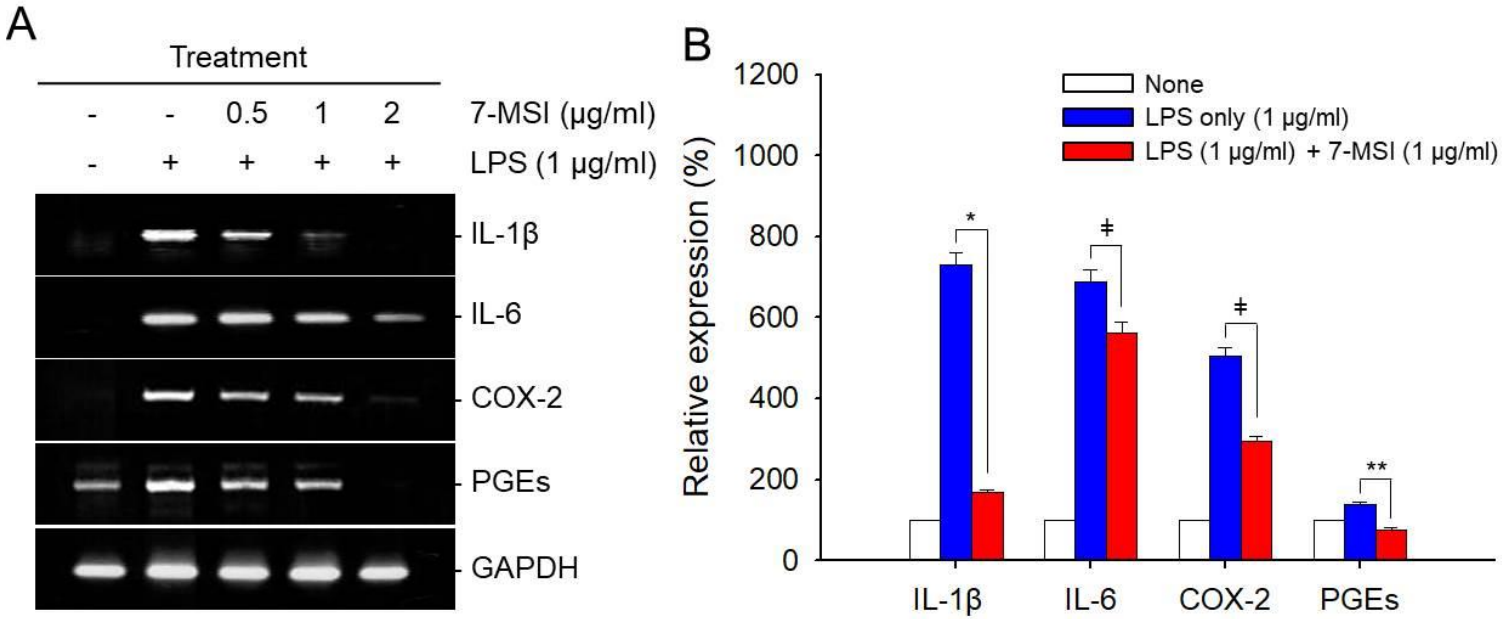


Fig. 15. Effects of 7-MSI on the transcription levels of various pro-inflammatory cytokines in Raw 264.7 cells. (A) Macrophage cells were treated with 7-MSI (0.5, 1, and 2 μg/ml) in the expression of lipopolysaccharide (1 μg/ml) and RT-PCRs were performed with total RNAs isolated from the corresponding experimental groups using primers specific for interleukin-1β, COX-2, interleukin-6, and PGEs. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

3-2-4 Effects of 7-MSI on IκB phosphorylation in Raw 264.7 cells

Nuclear factor kappa-light-chain-enhancer of activated B cells is located in the cytosol as in an inactive state by binding to IκB. However, the IκB protein is phosphorylated and then released from the complex when LPS is detected by TLR4, giving rise to NF-κB activation. The activated nuclear factor kappa-light-chain-enhancer of activated B cells translocates into nucleus to induce the expression of pro-inflammatory cytokines and enzymes (Kuo *et al.,* 2015). In this study, the phosphorylation of IκBα was analyzed in macrophage cells using Western blottings. As shown in Fig. 16, the IκBα was clearly degraded when the cells were treated with lipopolysaccharide (1 μg/ml). However, the degrees of IκBα degradation and phospho-IκBα production were significantly reduced by 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) treatments in lipopolysaccharide (1 μg/ml)-treated cells. When macrophage cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the presence of lipopolysaccharide (1 μg/ml) for 1 h, the expression level of phospho-IκBα was decreased by approximately 46%, as compared with that of the non-treated control. These results produced that 7-methylsulfonyl hepthyl isothiocynate can decrease the phosphorylation of Iκ Bα proteins to prevent the NF-κB activation in macrophage cells.

3-2-5 Confocal microscopic analysis of the inhibition of NF-κB activation by 7-MSI in Raw 264.7 cells

In this study, the inhibitory effect of 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) on the NF-κB activation was confirmed in lipopolysaccharide (1 μg/ml)-treated cells by Confocal microscopic analysis

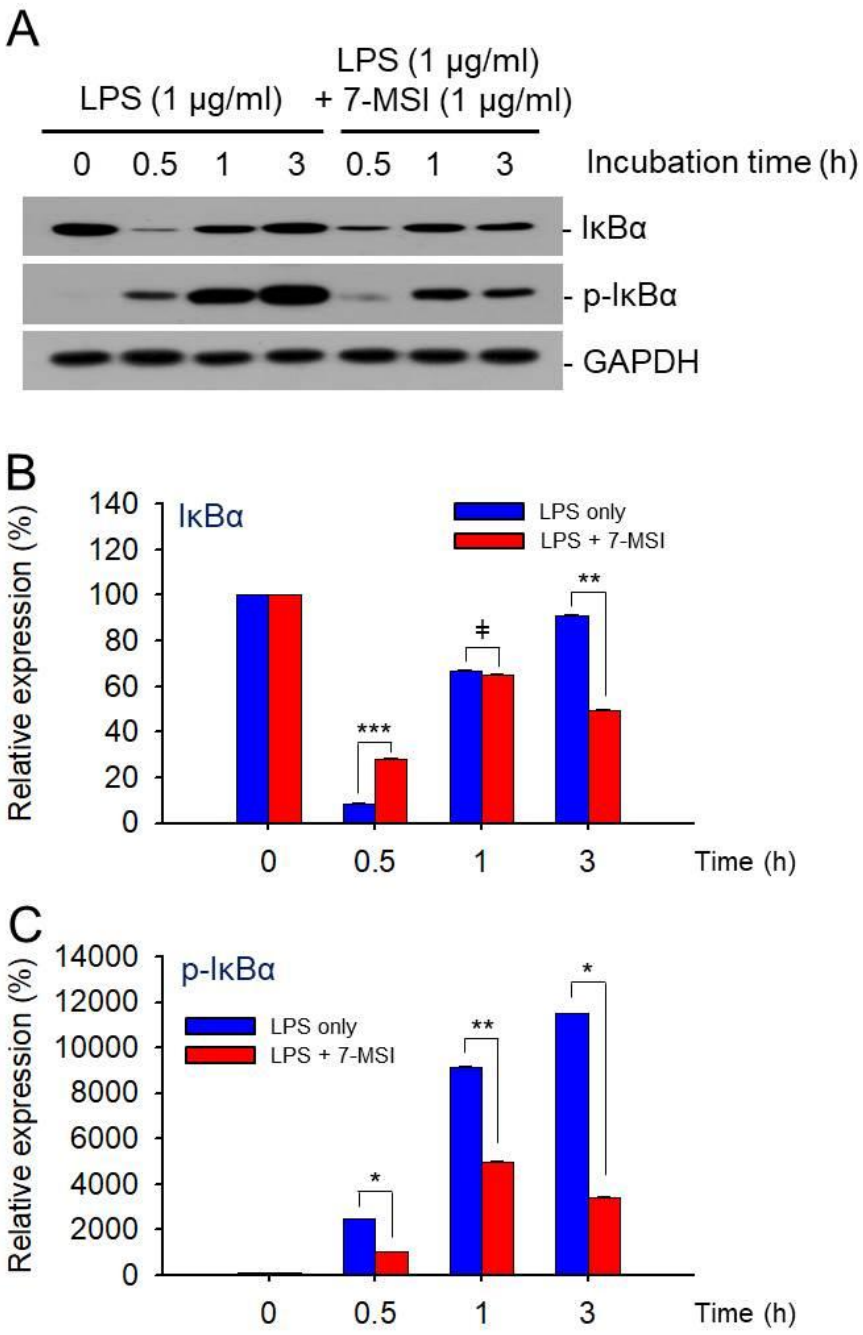


Fig. 16. Inhibition of the phosphorylation of IκB by 7-MSI in Raw 264.7 cells. (A) The cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml). Proteins were prepared and Western blottings were performed to examine the expression of IκBα, p-IκBα, and GAPDH. (B and C) Histograms showing the relative expression rates of IκB α and p-IκBα.

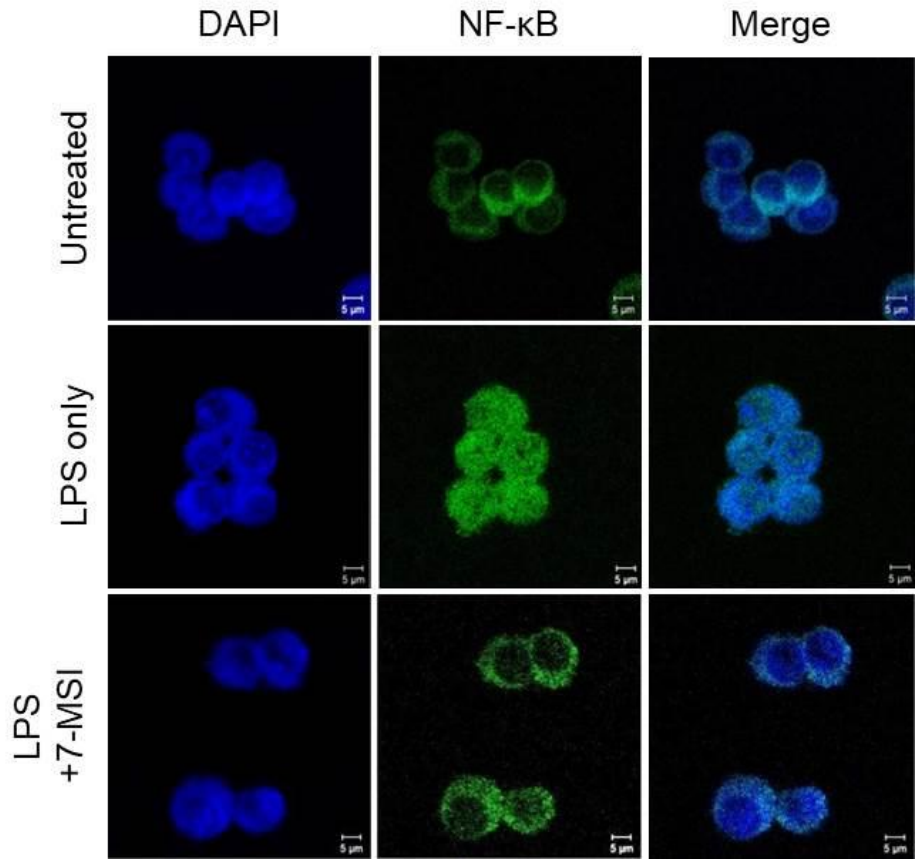


Fig. 17. Confocal microscopic analysis of the inhibition of LPS-induced NF-κB activation by 7-MSI in Raw 264.7 cells. The cells treated with lipopolysaccharide (1 μg/ml) alone or co-treated with lipopolysaccharide (1 μg/ml) and 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) were stained with anti-NF-κB p65 antibody labeled with fluorescein, from which the green fluorescences emitted were detected using confocal microscope.

using anti-NF-κB antibody labeled with fluorescein. As shown in Fig. 17, the confocal microscopy showed that the lipopolysaccharide-induced nuclear translocation of NF-κB proteins could be inhibited by 7-MSI treatment, allowing the protein remained in the cytoplasm. These results produced that 7-methylsulfonyl hepthyl isothiocynate can inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells signaling by inhibiting the phosphorylation of IκBα protein.

3-2-6. Effect of 7-MSI on NLRP3 inflammasome in RAW-ASC cells

The NLRP3 inflammasome consists of the adapter protein ASC, the NLR protein NLRP3, and pro-caspase-1. In this study, the expression levels of NLRP3 inflammasome components were analyzed in RAW-ASC cells using Western blottings with the corresponding antibodies. When the cells were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h. As shown in Fig. 18, the expression levels of NLRP3, ASC, and pro-caspase-1 were decreased by 9%, 80%, 34%, and 86%, in the cells treated with 2 μg/ml of 7-methylsulfonyl hepthyl isothiocynate compared to that of non 7-methylsulfonyl hepthyl isothiocynate-treated group cells treated with ATP (1 mM) in LPS (0.01 μ g/ml) primed cells. These results suggest that 7-methylsulfonyl hepthyl isothiocynate suppresses inflammasome formation in immune cells.

3-2-7. Effect of 7-MSI on the transcription levels of NLRP3 inflammasome in RAW-ASC cells

To analyze the effects of 7-methylsulfonyl hepthyl isothiocynate on

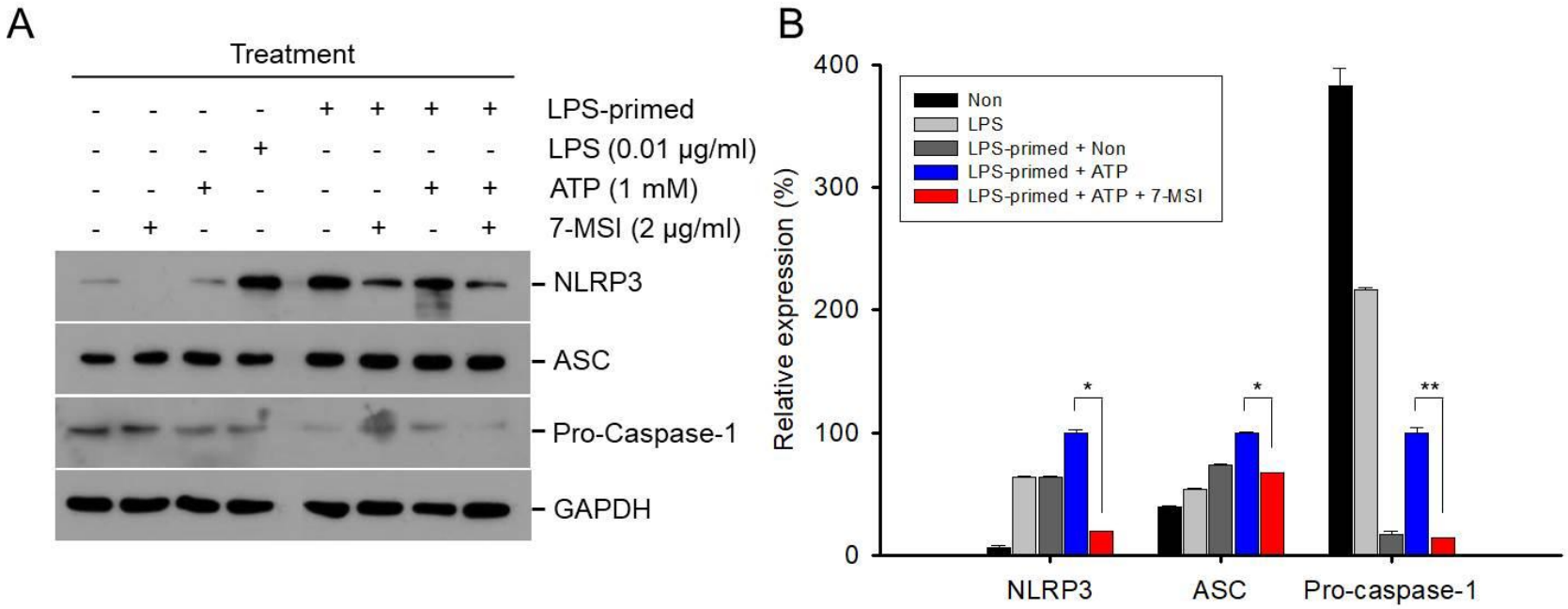


Fig. 18. Inhibition of the formation of NLRP3 inflammasome by 7-MSI in RAW-ASC cells. The cells were primed first with LPS (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (1 mM), from which the expressions of ASC, NLRP3, pro-Caspase-1, and GAPDH were determined by Western blottings. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

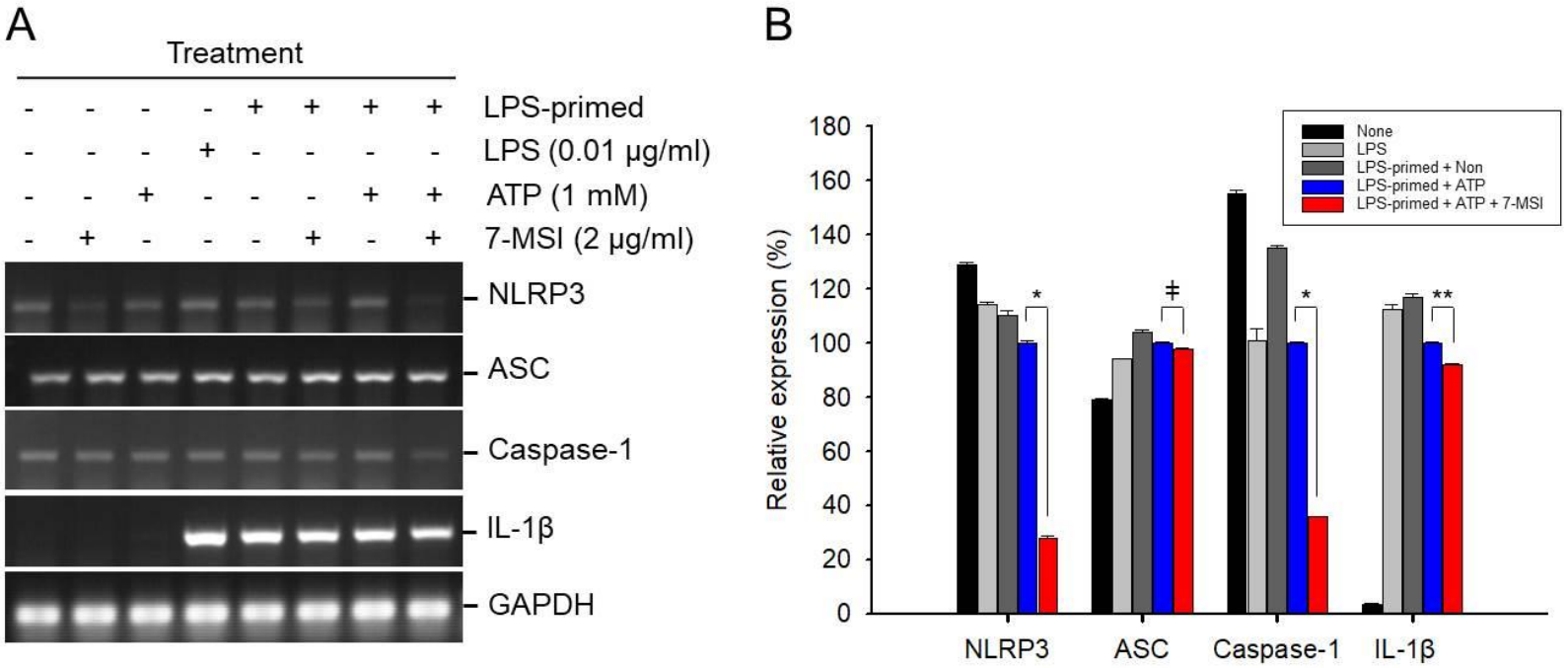


Fig. 19. Inhibition of the formation NLRP3 inflammasome by 7-MSI in RAW-ASC cells. RAW-ASC cells were primed first with LPS (0.01 μg/ml) for 3 h and then treated with 7-MSI (2 μg/ml) and ATP (1 mM) for 3 h, from which the expression levels of NLRP3, ASC, Caspase-1, interlukin-1β, and GAPDH were estimated by RT-PCR. The symbols “+” and “-” indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

inflammasome formation in skin immune cells, RAW-ASC cells were primed first with LPS (0.01 μg/ml) for 3 h and then co-treated with 7-MSI (2 μg/ml) and of ATP (1 mM). The results of RT-PCR showed that the transcription levels of ASC, NLRP3, Caspase-1, and interleukin-1β were decreased by approximately 3%, 72%, 65%, and 10%, respectively, in comparison with that of LPS-primed plus ATP group (Fig. 19). These results produced that 7-methylsulfonyl hepthyl isothiocynate can inhibit the LPS and ATP-induced inflammasome formation.

3-2-8. Confocal microscopic analysis of the inhibition of NLRP3 inflammasome formation by 7-MSI in RAW-ASC cells

To analyze the inhibition of the inflammasome formation by 7-MSI in skin immune cells, RAW-ASC cells were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-methylsulfonyl hepthyl isothiocynate (2 μg/ml) and ATP (1 mM). The cells were stained with anti-ASC and -Caspase-1 antibodies labeled with fluorescein and the green and red fluorescence emitted were detected with a confocal microscope. As shown in Fig. 20, the formation of inflammasome seemed to be suppressed in the cells treated primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 2 μg/ml of 7-methylsulfonyl hepthyl isothiocynate in the existence of ATP (1 mM). This result suggests that 7-methylsulfonyl hepthyl isothiocynate can inhibit the formation of inflammasomes.

3-2-9. Inhibitory effects of 7-MSI on caspase-1 activity in RAW-ASC cells

After priming, the inflammasome complex is formed and activated by

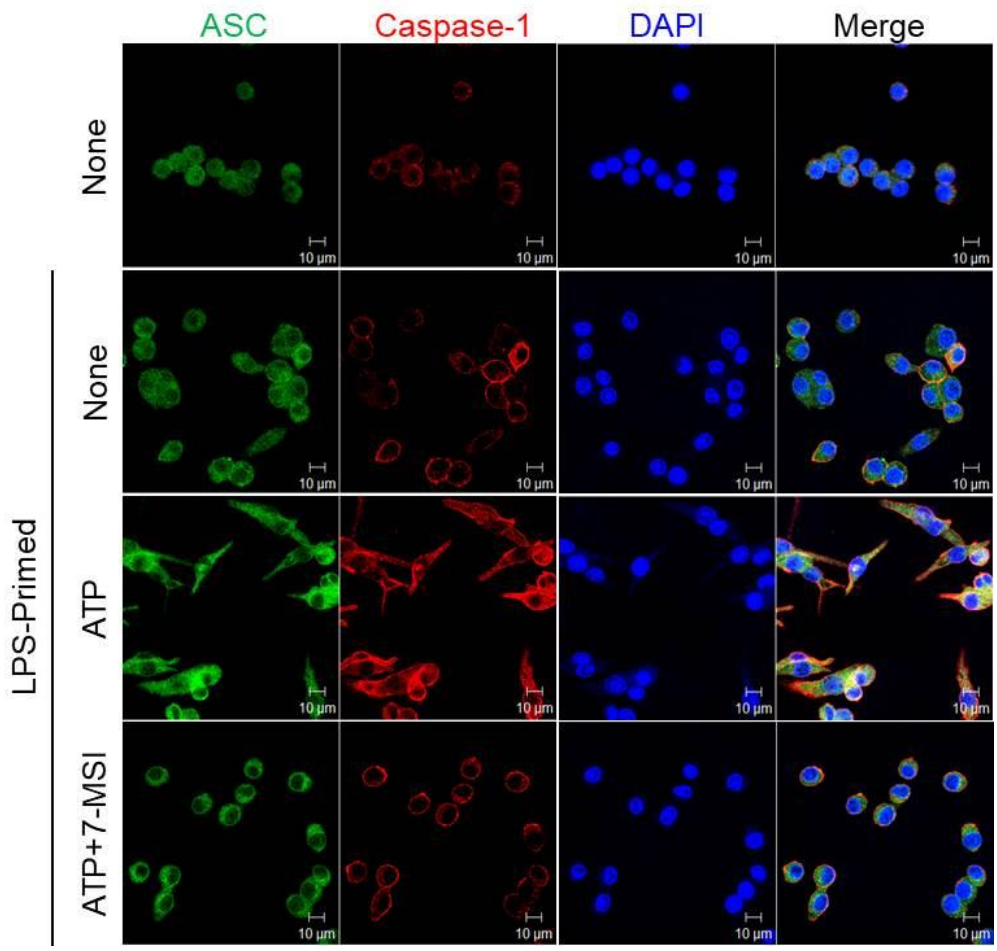


Fig. 20. Confocal microscopic analysis of the inhibition of inflammasome formation by 7-MSI in RAW-ASC cells. The cells were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-methylsulfonyl hepthyl isothiocynate (2 μg/ml) and ATP (1 mM). The cells were then stained with anti-ASC and -Caspase-1 antibodies labeled with fluorescein, from which the green and red fluorescences emitted were observed with a confocal microscope.

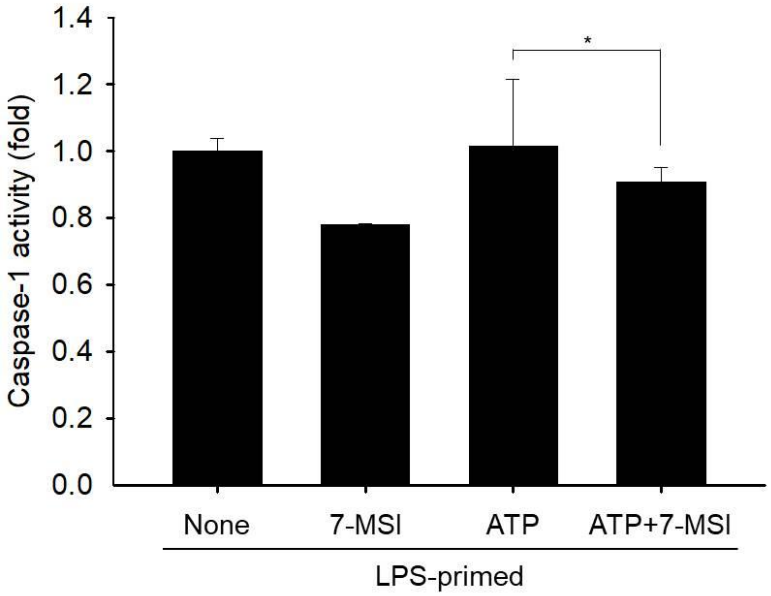


Fig. 21. Inhibition of caspase-1 activity by 7-MSI in RAW-ASC cells. Macrophage cells were primed first with LPS (0.01 μg/ml) and then co-treated with 7-methylsulfonyl hepthyl isothiocynate (2 μg/ml) and ATP (1 mM). Caspase-1 activity was measured using Caspase-1 assay kit. Results represent means ± S.D. of duplicate determinations from three independent experiments.

the second activation signal such as the mitochondrial ROS, ATP, and potassium efflux through ion channels. In the inflammasome generated by this second signal, caspase-1 is activated through autoproteolytic maturation, resulting in the release of interlukin-1β and interlukin-18. In this study, caspase-1 activity was analyzed in RAW-ASC cells using the caspase-1 activity assay. When the cells were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-methylsulfonyl hepthyl isothiocynate (2 μg/ml) and ATP (1 mM) for 3 h. As shown in Fig. 21, the caspase-1 activity was decreased by 10% in the cells treated with 2 μg/ml of 7-methylsulfonyl hepthyl isothiocynate compared to that of the group without 7-methylsulfonyl hepthyl isothiocynate.

3-2-10. Inhibitory effects of 7-MSI on IL-1β production in RAW-ASC cells

To analyze the effect of 7-methylsulfonyl hepthyl isothiocynate on IL-1β release and inflammasome formation in skin immune cells, ELISA was performed with RAW-ASC cells that were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-methylsulfonyl hepthyl isothiocynate (2 μ g/ml) and ATP (1 mM). ELISA data showed that the production amount of IL-1β was reduced by about 80% in 7-methylsulfonyl hepthyl isothiocynate-treated group (Fig. 22). These results produced that 7-methylsulfonyl hepthyl isothiocynate can inhibit IL-1β release by suppressing inflammasome formation.

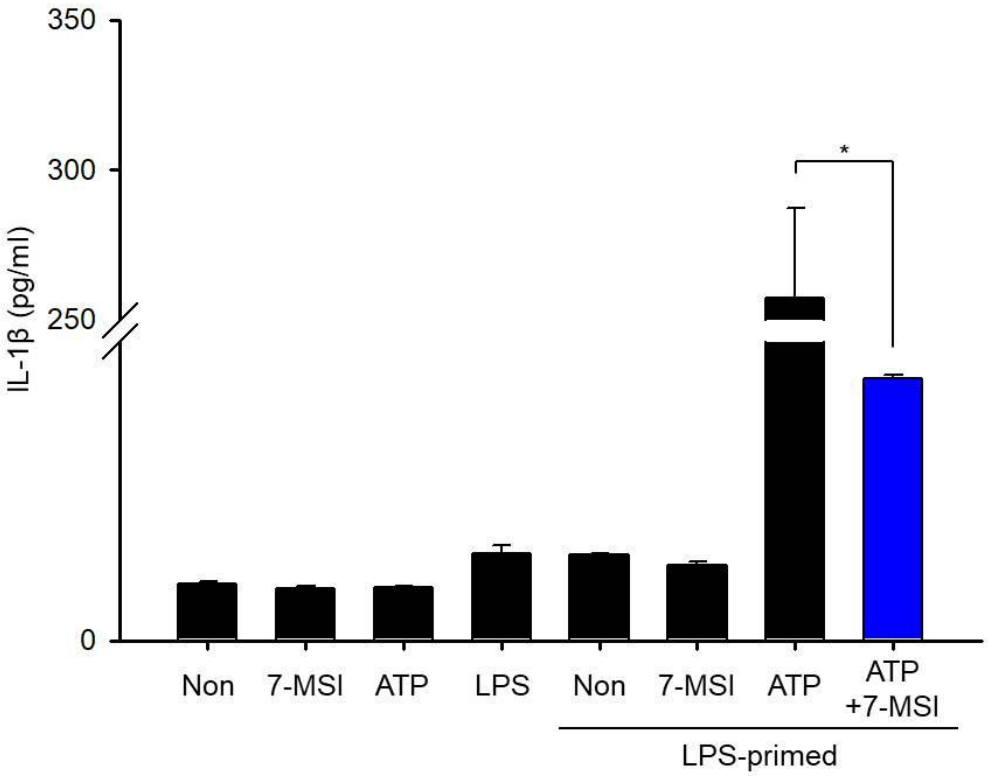


Fig. 22. Effects of 7-MSI on IL-1β production in RAW-ASC cells. The cells were primed first with LPS (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (2 mM), from which IL-1β concentrations in the culture supernatants were measured using a IL-1β-specific ELISA kit. Results represent means ± S.D. of duplicate determinations from three independent experiments.

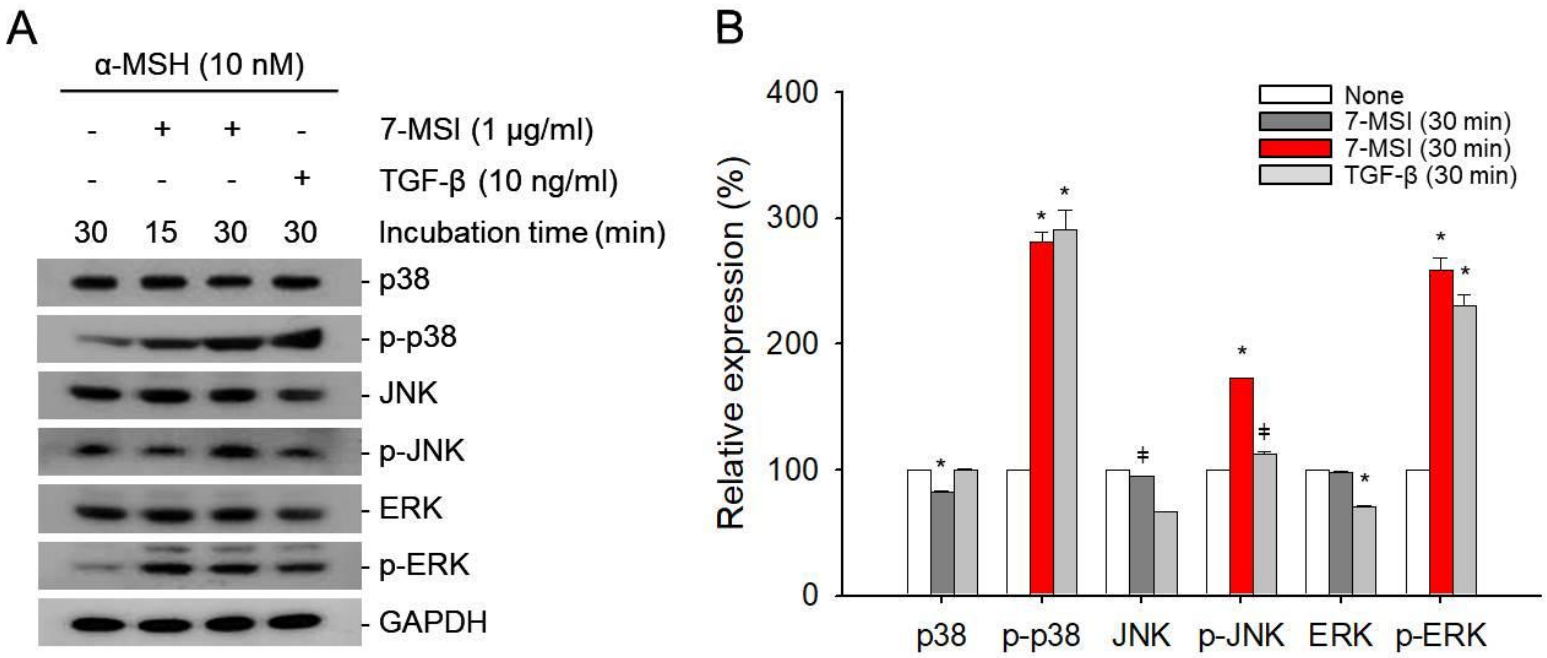


Fig. 23. Activation of MAPK signaling pathway by 7-MSI in B16-F1 cells. (A) Western blottings for the expressions of JNK, p-JNK, p38, p-p38, ERK, p-ERK, and GAPDH in melanoma cells treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml). The symbols “+” and “-” indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

3-3. Inhibitory effects of 7-MSI on melanogenesis and inflammatory response through the cellular activation of autophagy

3-3-1. Effect of 7-MSI on MAPK signaling in B16-F1 cells

It has been known that MAPK signaling regulates the induction of autophagy and especially the activated ERK can lead the maturation of autophagic vacuoles (Martin *et al.,* 2006). The effects of 7-methylsulfonyl hepthyl isothiocynate on activation of MAPK pathway were also investigated in melanoma cells using Western blottings with antibodies raised against MAPK-related proteins. When melanoma cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of α-MSH (10 nM), the expression levels of p-JNK, p-ERK, and p-p38 were increased by roughly 72%, 158%, and 181%, respectively, as in comparison with those of control cells (Fig. 23). These results produced that 7-methylsulfonyl hepthyl isothiocynate can induce up-regulation of the MAPK pathway in melanoma cells.

3-3-2. Activation of cellular autophagy by 7-MSI in B16-F1 cells

The activated mTORs suppress autophagy. The Beclin-1-release through the activation of MAPKs induces autophagy activation. The activation of autophagy begins with the formation of phagophores (also called isolation membranes). The phagophores expand and capture cargos inside and then form autophagosome to be fused with a lysosome (Zhou *et al*., 2015). The Atg complex and LC3-II (also known as LC3-II that is a prominent marker

molecule routinely used for the detection of autophagosome) bind to the phagophore membrane and the autophagosome is formed. The resulting autophagosomes are fused to lysosomes, in which cargos are degraded by hydrolases (Zhang *et al.,* 2008). To investigate the activation of autophagy system by 7-methylsulfonyl hepthyl isothiocynate, the protein expression levels of autophagy factors were analyzed in melanoma cells treated with 1 μg/ml of 7-methylsulfonyl hepthyl isothiocynate in the existence of α-MSH (10 nM) using Western blottings. As shown in Fig. 24, the expression levels of Beclin-1, LC3, and Atg12 were increased by 34%, 46%, and 50%, respectively, compared to those of the non-treated control. However, the expression level of phospho-mTOR (a negative regulator of the autophagy system) was decreased by about 52% after treatment with 7-methylsulfonyl hepthyl isothiocynate. These results produced that 7-methylsulfonyl hepthyl isothiocynate can increase the expression of autophagy-related proteins in melanoma cells.

3-3-3. Formation of autophagosomes by 7-MSI in B16-F1 cells

The activation of autophagy begins with the formation of phagophores (also called isolation membranes). The phagophores expand and capture cargos inside and then form autophagosome to be fused with a lysosome (Zhou *et al*., 2015). The Atg complex and LC3-II (also known as LC3-II that is a prominent marker molecule routinely used for the detection of autophagosome) bind to the phagophore membrane and the autophagosome is formed. The resulting autophagosomes are fused to lysosomes, in which cargos are degraded by hydrolases (Zhang *et al.,* 2008). In this study, the ability of 7-methylsulfonyl hepthyl isothiocynate to form autophagosomes was observed by confocal microscopy. As shown in Fig. 25, the autophagosomes could be formed in the cells treated with 7-methylsulfonyl hepthyl isothiocyna-

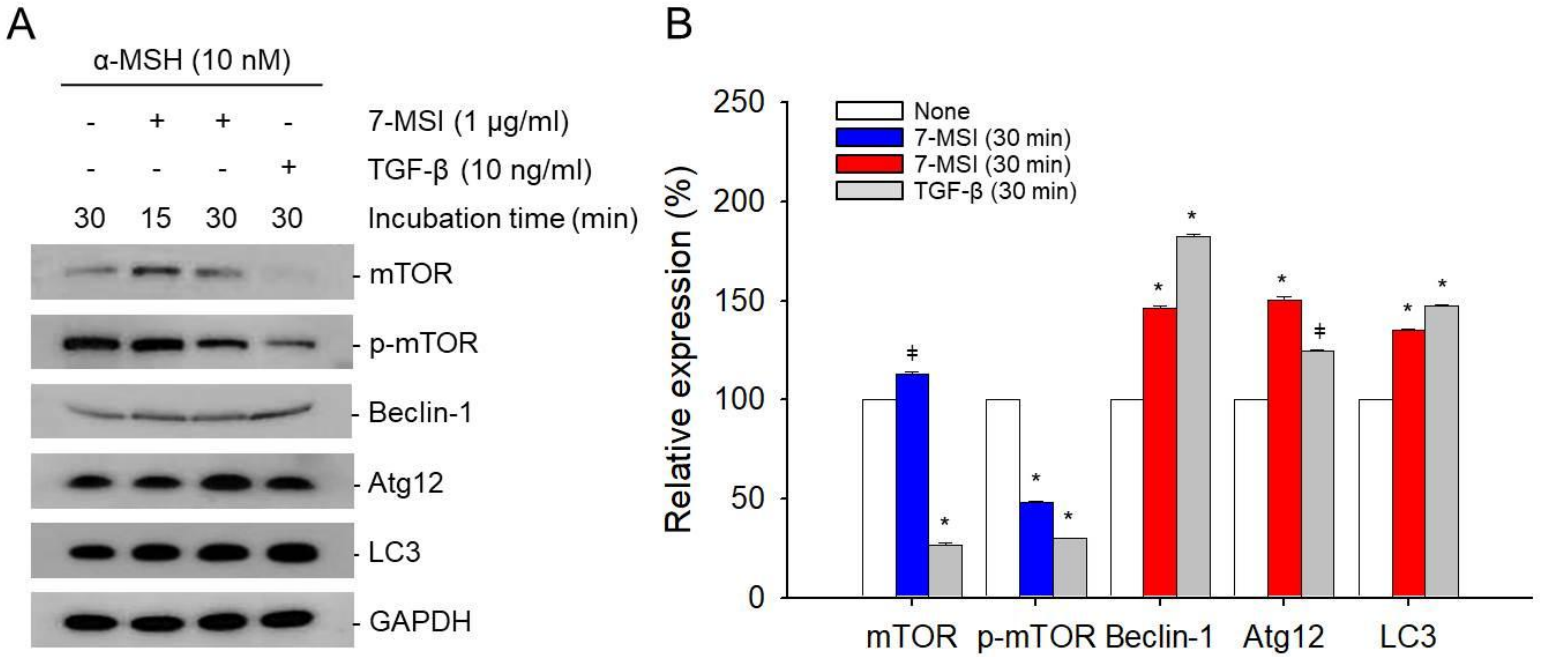


Fig. 24. Activation of cellular autophagy by 7-MSI in B16-F1 cells. (A) The cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of α-MSH (10 nM). Proteins were prepared and the expressions of mTOR, p-mTOR, Beclin-1, LC3, Atg12, and GAPDH were determined by Western blottings. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

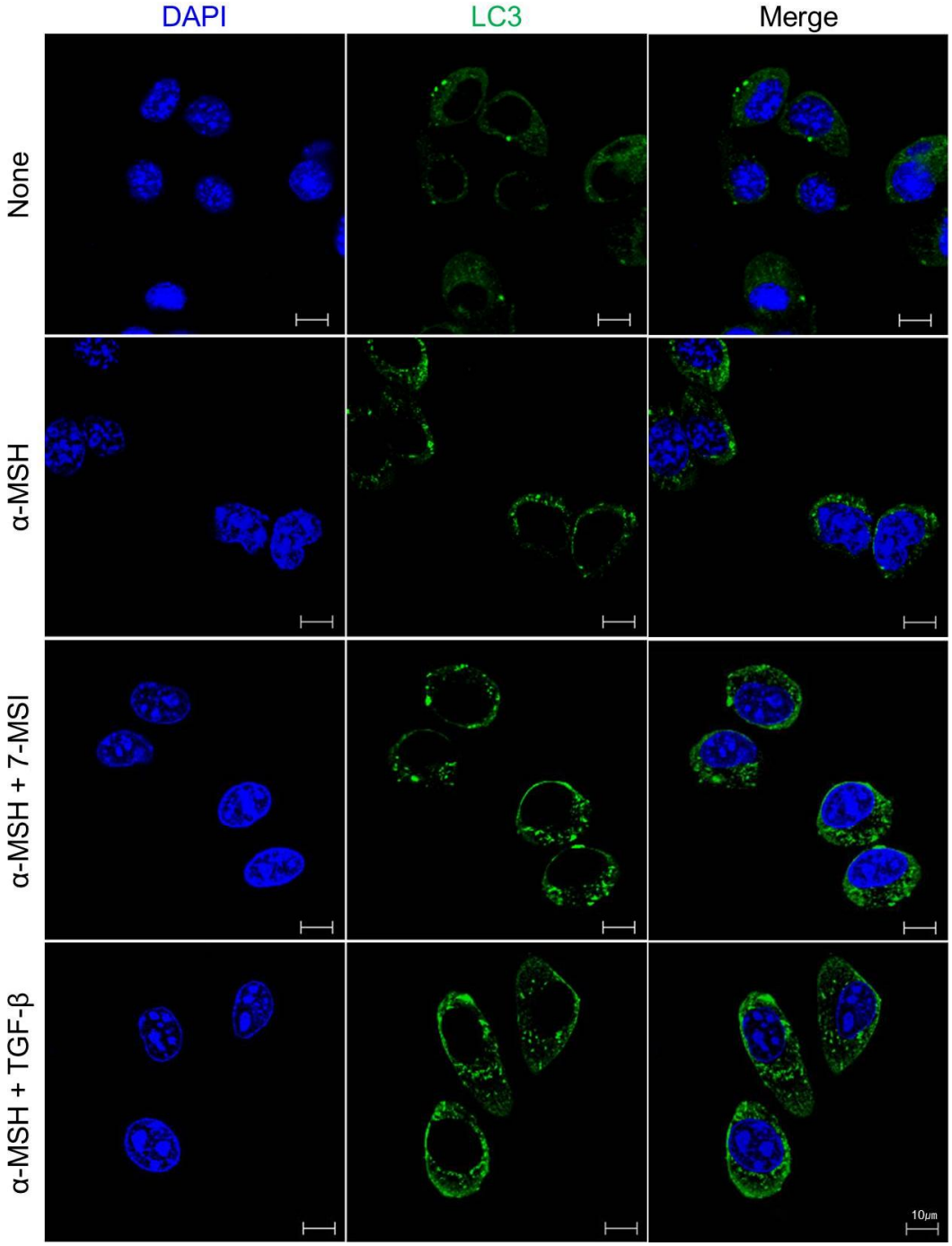


Fig. 25. Confocal microscopic analysis of the induction of autophagy 7-MSI in B16-F1 cells. Melanoma cells treated with α-MSH (10 nM) alone and 7-MSI (1 μg/ml) or TGF-β (10 ng/ml) in the presence of α -MSH (10 nM) were stained with anti-LC3 antibody labeled with fluorescein and the fluorescences were detected with confocal microscope. The cells were also stained with DAPI to show nuclei.

te (1 μg/ml), TGF-β (10 ng/ml) or α-MSH (10 nM). This result suggests that 7-methylsulfonyl hepthyl isothiocynate can lead the production of autophagosome.

3-3-4. Effect of siRNAs against Atg5 on the production of melanin synthesis

All the results above prove that 7-methylsulfonyl hepthyl isothiocynate could activate the autophagy by up-regulating the autophagy-associated proteins. In addition, the compound inhibits melanogenesis by down-regulating melanogenesis-related proteins. However, it was still necessary to prove that 7-methylsulfonyl hepthyl isothiocynate could activate the autophagy system that eventually leads to the diminution of melanin product. Therefore, the reverse genetic approaches using small interfering RNAs (siRNAs) against Atg5 mRNA were applied to reveal a association between the activation of the autophagy and the inhibition of the production of melanin by 7-methylsulfonyl hepthyl isothiocynate (Fig. 26). When non-transfected melanoma cells were treated with 7-methylsulfonyl hepthyl isothiocynate (0.05, 0.1, 0.5, 1, or 2 μg/ml) for 72 h, there were clear suppresses in melanin product in a dose-dependent manner. However, when the cells were transfected with siRNAs for Atg5, the inhibitory effect of 7-methylsulfonyl hepthyl isothiocynate on melanogenesis was reduced. These results clearly show that 7-methylsulfonyl hepthyl isothiocynatecan decrease melanin production in melanoma cells by the activation of the autophagy.

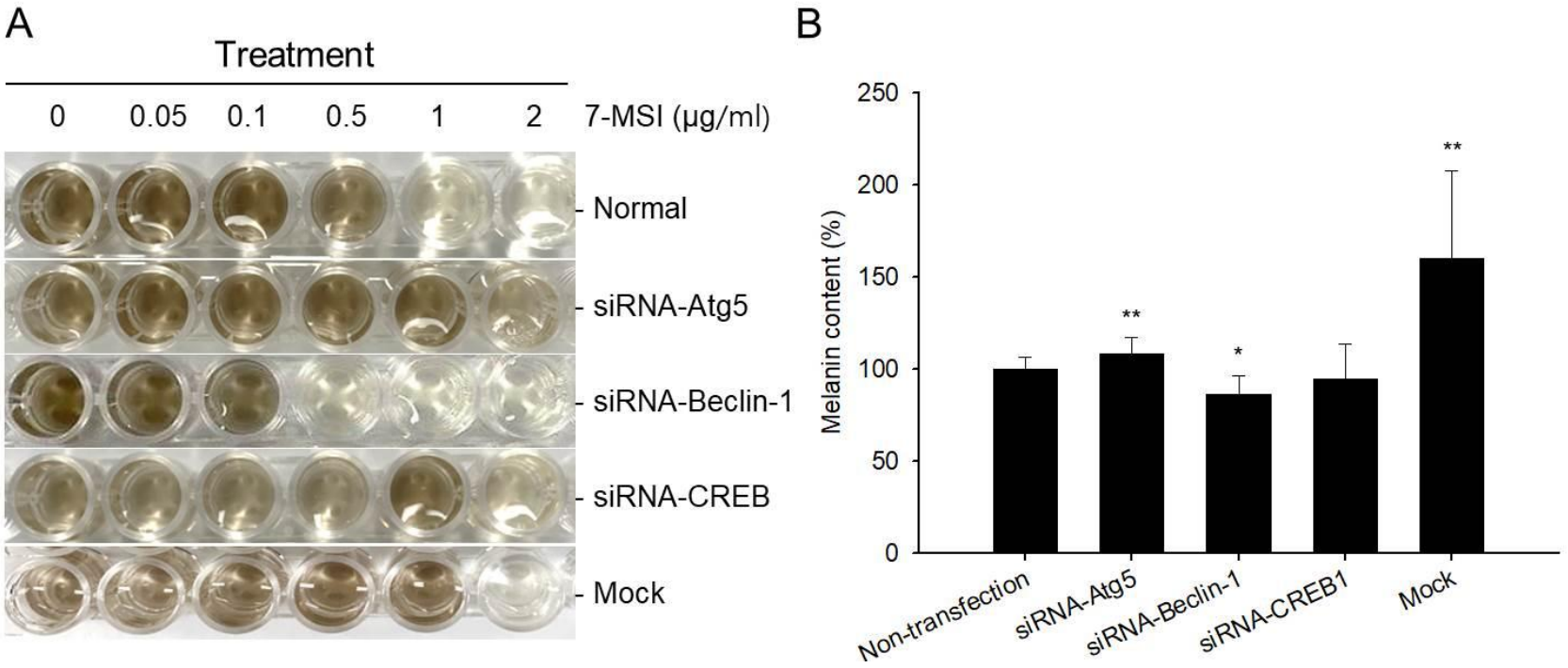


Fig. 26. Effects of siRNAs against Atg5, Beclin-1, and CREB on the melanin production in B16-F1 cells. The cells were non-transfected or transfected with siRNAs specific to Atg5, Beclin-1, and CREB. (A) Photographs showing the inhibitory effects of the corresponding siRNAs. (B) The melanin contents were calculated by measuring the absorbance at 490 nm.

3-3-5. Effects of 7-MSI on MAPK signaling pathway in Raw 264.7 cells

The effects of 7-methylsulfonyl hepthyl isothiocynate on activation of MAPK pathway were also investigated in macrophage cells using Western blottings. When macrophage cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml) for 15 or 30 min, the expression levels of p-JNK, p-ERK, and p-p38 were increased by roughly 829%, 1,486%, and 89%, in comparison with that of lipopolysaccharide only (Fig. 27). These results further produced that 7-methylsulfonyl hepthyl isothiocynate can induce the up-regulation of the MAPK signaling in macrophage cells.

3-3-6. Activation of cellular autophagy by 7-MSI in Raw 264.7 cells

When macrophage cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml), the expression levels of Beclin-1, LC3, and Atg12 were increased by roughly 377%, 27%, and 77% (Fig. 28). However, the expression level of phospho-mTOR was decreased by about 34% with 7-methylsulfonyl hepthyl isothiocynate treatment. These results show that 7-methylsulfonyl hepthyl isothiocynate can increase the expression of autophagy-associated proteins in macrophage cells.

3-3-7. Formation of autophagosomes by 7-MSI in Raw 264.7

In this study, the ability of 7-methylsulfonyl hepthyl isothiocynate to

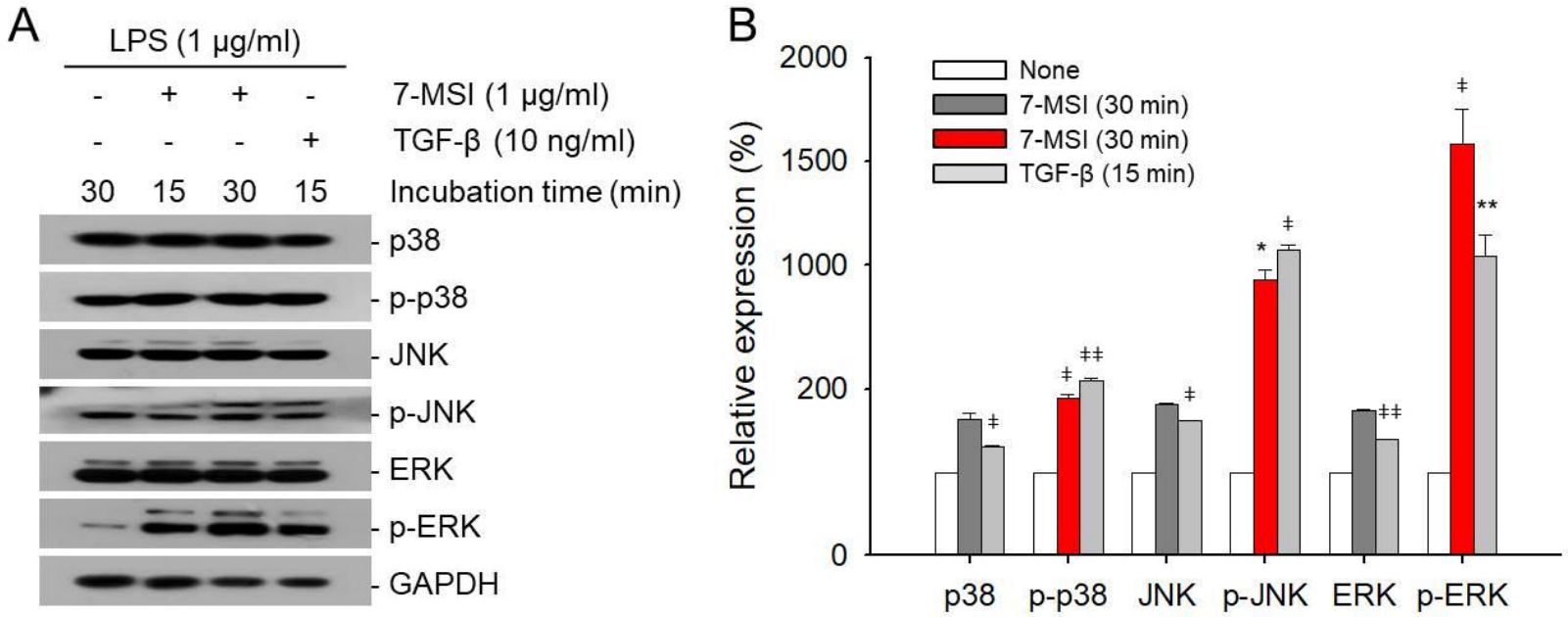


Fig. 27. Activation of the MAPK signaling pathway by 7-MSI in Raw 264.7 cells. (A) The cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml), from which the expressions of MAPK-related proteins and GAPDH were determined by Western blottings. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

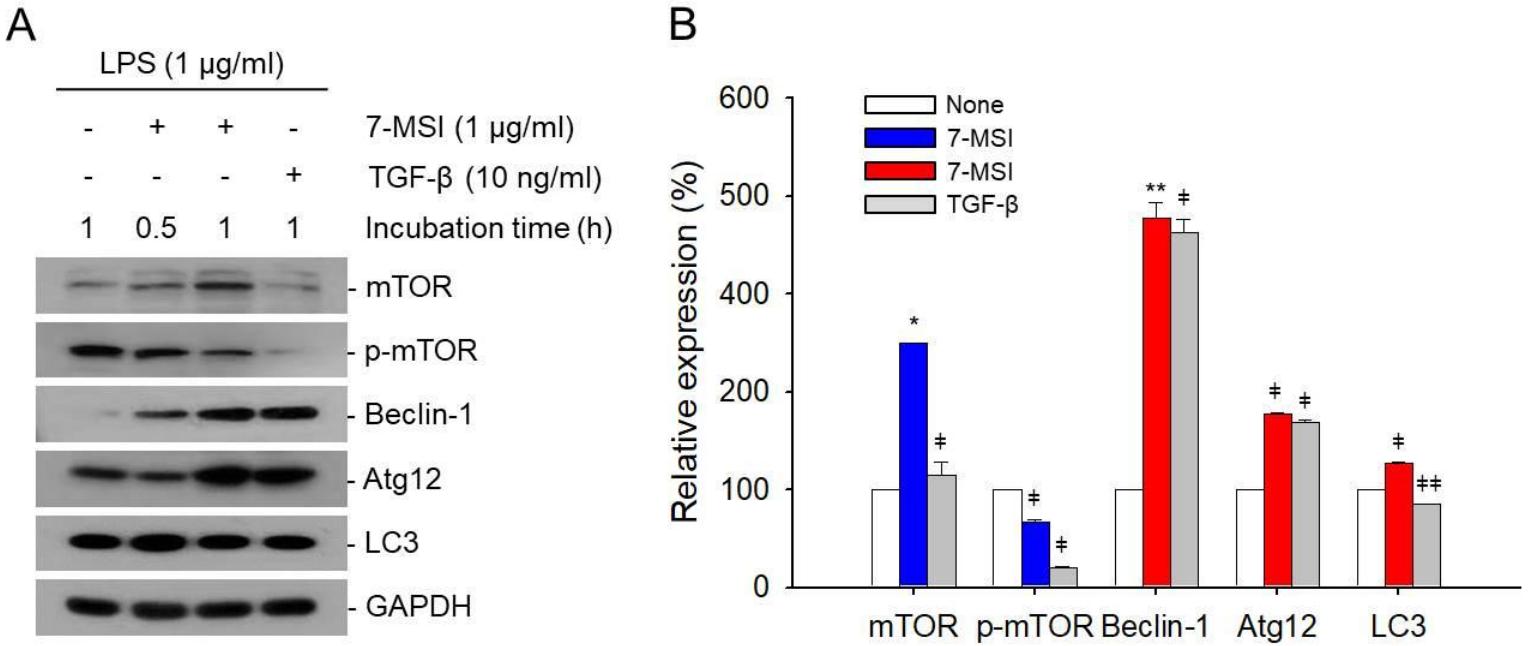


Fig. 28. Activation of cellular autophagy by 7-MSI in Raw 264.7 cells. (A) The cells were treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) in the existence of lipopolysaccharide (1 μg/ml), from which the expressions of mTOR, p-mTOR, autophagy-related proteins, and GAPDH were determined by Western blottings. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates from 1 h group of panel (A).

form autophagosomes was observed using immunostaining with anti-LC3 antibody. As shown in Fig. 29, the autophagosomes could form in the cells treated with 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml), TGF-β (10 ng/ml) or lipopolysaccharide (1 μg/ml). These results produced that 7-methylsulfonyl hepthyl isothiocynate can lead the production of autophagosome.

3-3-8. Effect of siRNAs against Atg5 and Beclin-1 on the TNF-α production in RAW-ASC cells

All the results above show that 7-methylsulfonyl hepthyl isothiocynate could activate the autophagy system by up-regulating the autophagy-associated proteins, with the suppression of the production of inflammatory cytokines and the formation of inflammasome. However, it was still necessary to confirm that 7-methylsulfonyl hepthyl isothiocynate could activate the autophagy system that finally leads to the lowering of inflammatory response. Therefore, the reverse genetic approaches using siRNAs against Atg5 and Beclin-1 mRNAs were applied to examine the association between the activation of the autophagy and the inhibition of inflammatory response by 7-methylsulfonyl hepthyl isothiocynate. RAW-ASC cells were treated with lipopolysaccharide (0.01 μg/ml) and 7-methylsulfonyl hepthyl isothiocynate (2 μg/ml), TNF-α production was analyzed. The results show that the production in level of TNF-α was reduced by 26% in non-transfected cells, however, it was rather increased by 5% in Beclin-1 siRNA-transfected cell (Fig. 30).

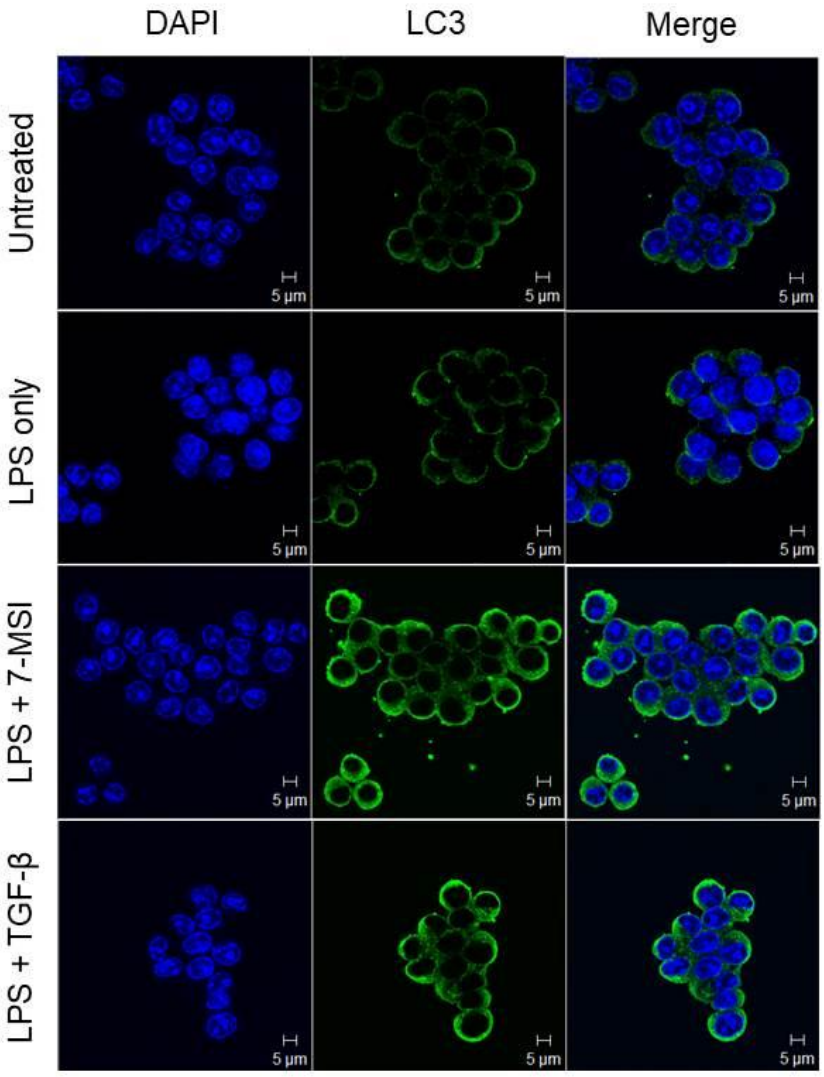


Fig. 29. Confocal microscopic analysis of the induction of autophagy by 7-MSI in Raw 264.7 cells. Macrophage cells treated with LPS (1 μg/ml) or co-treated with lipopolysaccharide (1 μg/ml) and 7-methylsulfonyl hepthyl isothiocynate (1 μg/ml) were stained with anti-LC3 antibody labeled with fluorescein and the green fluorescences emitted were detected with confocal microscope.

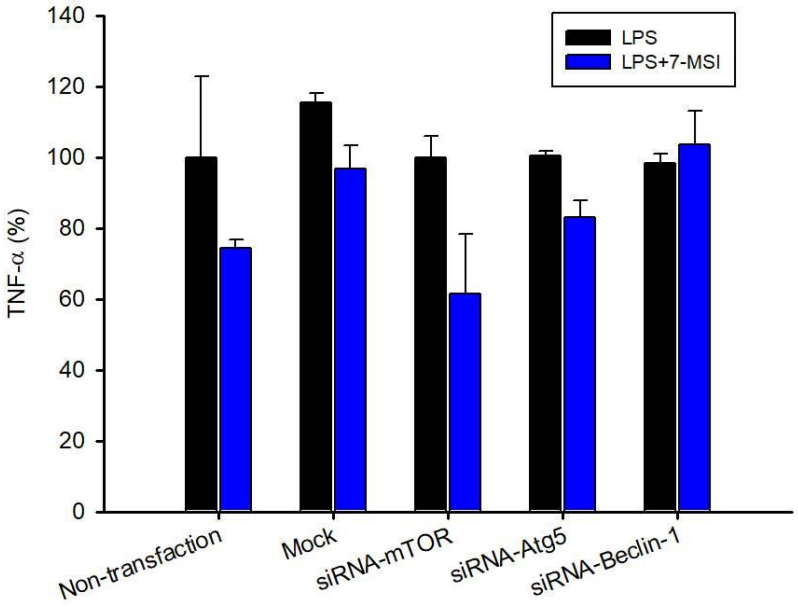


Fig. 30. Effects of Atg5- and Beclin-1-knockdowns on the production of TNF-α. The cells were transfected with siRNAs specific for Beclin-1, mTOR, Atg5 or a non-specific control siRNA (Mock). The transfected RAW-ASC cells were then first primed with LPS (0.01 μg/ml) and treated with 7-MSI (2 μg/ml) and ATP (1 mM), from which TNF-α density in the culture supernatants were measured using an ELISA kit specific for TNF-α.

3-3-9. Effect of siRNAs against Atg5 and Beclin-1 on the NLRP3 inflammasome in RAW-ASC cells

To investigate the association between the activation of the autophagy and the inhibition of inflammasome formation by 7-MSI, Western blotting and ELISA for IL-1β were performed with RAW-ASC cells were primed first with lipopolysaccharide (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (1 mM). As shown in Fig. 31, the expression levels of NLRP3 and ASC were increased by 20% and 48%, respectively, in the cells transfected with siRNAs for Beclin-1, in comparison with in non-transfected cells. The expression levels of NLRP3 and ASC were also increased by 20% and 48%, respectively, in the cells transfected with siRNAs for Beclin-1 compared to non-transfected cells. In addition, the amount of IL-1β released was transfection with siRNA-Atg5 increased by 5% in the cells transfected with Atg5 siRNA, compared to non-transfected cells (Fig. 32).

Taken together, the results suggest that 7-MSI can exhibit various effects such as skin whitening, anti-skin aging, and anti-inflammation through by 1) inhibition of melanin synthesis; 2) inhibition of inflammation through the reduction of expression of pro-inflammatory cytokines and the formation of inflammasomes; and 3) activation of the autophagy system by MAPK signaling (Fig. 33). In conclusion, all results current by this study prove that 7-MSI has a potential to be developed as a cosmetic material for multifunctional skin anti-aging and skin disease treatment.

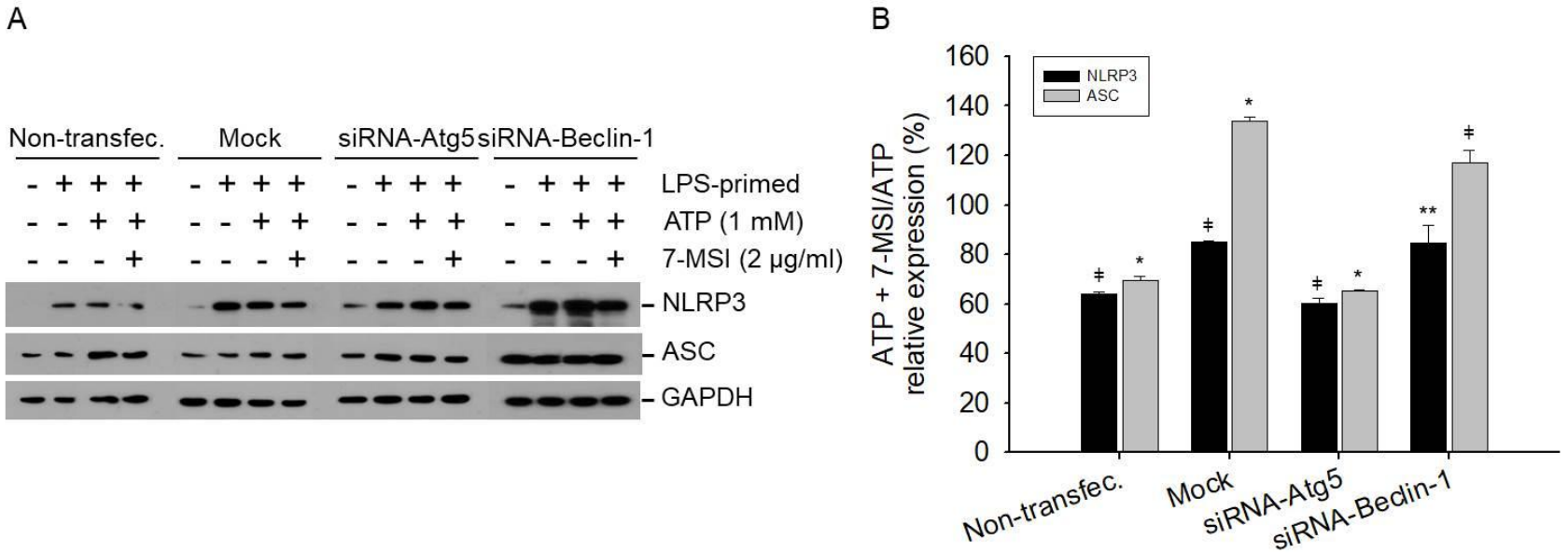


Fig. 31. Effects of Atg5- and Beclin-1-knockdowns on NLRP3 inflammasome. RAW-ASC cells were transfected with siRNAs specific for Beclin-1, Atg5 or a non-specific control siRNA (Mock). The transfected RAW-ASC cells were primed first with LPS (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (2 mM). Proteins were prepared and the expression levels of NLRP3 and ASC were examined by Western blottings. The symbols "+" and "-" indicate the addition and the omission of corresponding additives, respectively. (B) Histograms showing the relative expression rates.

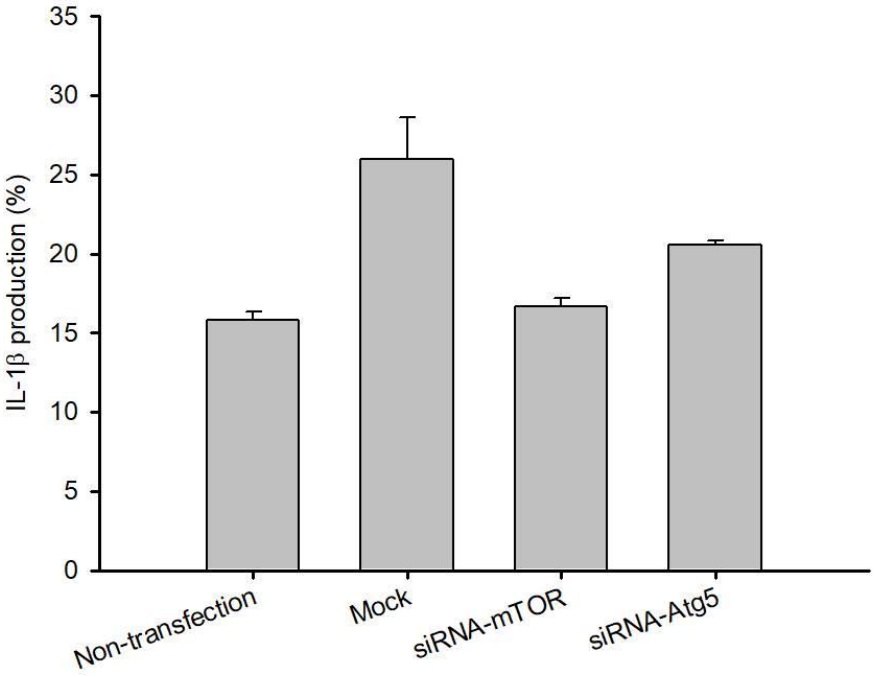


Fig. 32. Effects of mTOR- and Atg5-knockdowns on the production of IL-1β. RAW-ASC cells were transfected with siRNAs specific for Atg5, mTOR or a non-specific control siRNA (Mock). The transfected RAW-ASC cells were first primed with LPS (0.01 μg/ml) and then treated with 7-MSI (2 μg/ml) and ATP (1 mM), from which IL-1β density in the culture supernatants were measured using an ELISA kit specific for IL-1β.

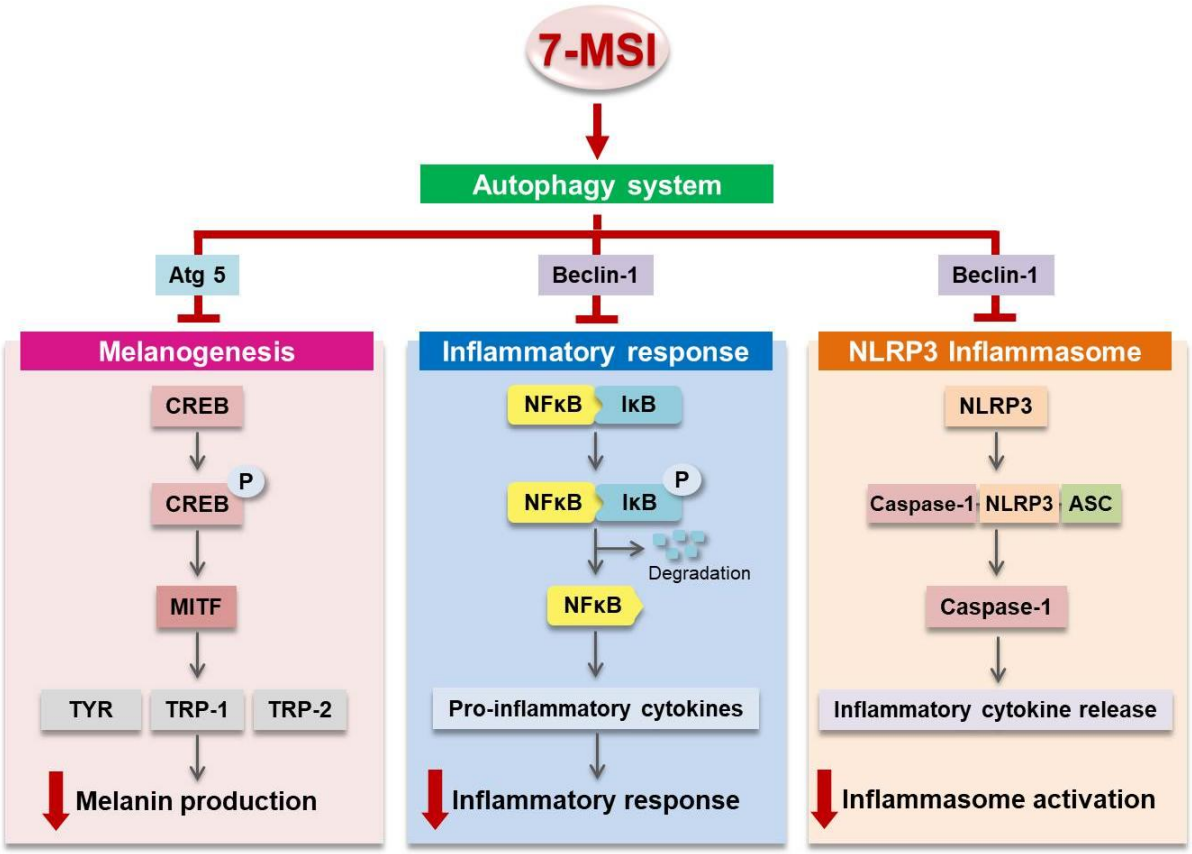


Fig. 33. Schimatic summary of effects of 7-MSI on skin whitening, anti-inflammation and autophagy activation. The compound 7-MSI shows various inhibitory effects on melanin production and inflammation through the activations of NF-κB and autophagy. 7-MSI can inhibit the melanin synthesis by inhibiting melanogensis, leading to skin whitening. It also can suppress cellular inflammatory response by inhibiting the production of pro-inflammatory cytokines and the activation of inflammasome as well.

4. 적 요

피부미백, 항염증 및 autohagy 활성화에 미치는 7-methylsulfonylheptyl isothiocyanate의 영향

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피부는 흡연, 자외선, 환경 오염과 유전적 배경 등 다양한 요인에 의해 영향을 받는다. 이러한 요인들은 함께 작용하여 멜라닌 과색소 침착, 주름, 피부 탄력 저하 및 처짐을 특징으로 하는 피부노화를 유발한다. 피부는 세포 내 노폐물이나 외부 침입 물질들을 분해하는 자가포식(autophagy) 시스템을 활성화시켜 세포의 항상성을 유지한다. 또한 자가포식의 조절은 염증성 질병과 노화에도 영향을 미치는 것으로 알려져 있다. 한편, NLRP3 염증소체(inflammasome)는 선천성 면역조절에 매우 중요한 역할을 하는 것으로 알려져있다. 본 연구에서 사용한 7-MSI (7-methylsulfonylhepthyl isothiocynate)는 십자화과 식물에 풍부한 파이토케미컬(phytochemical) 중 하나로써 항산화, 항염증, 항암 효과가 있는 것으로 알려져 있으나 피부 미백 및 항노화에 대한 효과는 아직 밝혀지지 않았다. 따라서 본 연구에서는 7-MSI의 피부 미백, 항노화 및 항염증에 미치는 효과를 멜라닌 생성, 염증성 사이토카인 생성, inflammasome 형성 및 autophagy 활성화를 분석하여 규명하고자 하였다. α-MSH (α-melanocyte-stimulatng hormone)를 처리하여 멜라닌 생성을 촉진시킨 흑색종 세포(B16-F1)에 7-MSI (0.5 ㎍/ml)를 72시간 처리한 결과, α-MSH만 처리한 세포에 비해 멜라닌 생성이 약 63% 감소됨을 확인하였다. 또한 7-MSI에 의한 멜라닌 생성 관련 단백질들의 발현 양상을 Western blotting으로 분석한 결과, CREB, p-CREB, MITF, tyrosinase 및 TRP1의 발현량이 모두 감소함을 확인하였다. 이러한 결과는 7-MSI가 멜라닌

생성의 생화학적 경로를 직접 억제함으로써 멜라닌 생성을 감소시킬 수 있음을 시사하는 것이다. 한편, 염증성 사이토카인 생성에 대한 7-MSI의 억제 효과를 대식세포주인 Raw 264.7 세포에서 조사하였다. ELISA (enzyme-linked immunosorbent assay)와 RT-PCR (reverse transcription-PCR)을 수행한 결과, 7-MSI는 interleukin-1β, interleukin-6, COX-2 및 PGE 등과 같은 전염증성 사이토카인 및 중재자들의 발현을 모두 억제할 뿐만 아니라 TNF-α 생성량도 감소시킴을 확인하였다. 또한 Western blotting (IκBα와 p-IκBα에 대한 항체 사용) 및 면역형광(NF-κB에 대한 항체 사용)을 통해 7-MSI가 NF-κB의 활성화를 억제하여 전염증성 사이토카인들의 생성을 억제할 수 있음을 확인하였다. 본 연구에서는 또 다른 대식세포주인 RAW-ASC 세포에서 inflammasome 형성에 대한 7-MSI 효과를 조사하였다. RAW-ASC 세포에 LPS (0.01 μg/ml)를 3시간 동안 처리한 후, ATP (1 mM) 및 7-MSI (2 μg/ml)를 3시간 동안 처리하였을 때, NLRP3, ASC 및 caspase-1과 같은 inflammasome 구성요소들의 발현이 감소됨을 Western blotting으로 확인하였으며, IL-1β 생성은 약 80% 감소됨을 ELSIA로 확인하였다. 이러한 결과는 7-MSI가 면역세포에서 inflammasome 형성을 억제할 수 있음을 시사한다. 본 연구에서는 또한 MAPK 신호전달을 통한 autophagy 활성화에 미치는 7-MSI의 영향을 조사하였다. Western blotting 결과, 7-MSI에 의해 p-p38, p-ERK 및 p-JNK를 포함한 MAPK-관련 단백질의 발현이 증가됨을 확인하였다. 또한, 7-MSI는 mTOR (autophagy의 음성조절자)의 발현을 감소시킨 반면, Beclin-1, Atg12 및 LC3-II의 발현은 증가시킴을 확인하였다. 면역형광 결과는 7-MSI에 의해 autophagosome의 형성이 증가됨을 보여주었다. 이러한 결과들은 7-MSI가 MAPK의 활성화를 통해 autophagy 시스템을 활성화시킬 수 있음을 시사하는 것이다. 본 연구에서는 Atg5 및 Beclin-1에 대한 small interfering RNA (siRNA)를 도입한 흑색종(B16-F1) 및 대식세포(RAW-ASC) 세포에서 atuophagy 시스템과 염증반응과의 관련성을 조사하였다. Atg5 siRNA가 도입된 B16-F1 세포에서는 멜라닌 생성에 대한 7-MSI의 억제 효과가 감소됨을 확인하였으며, Becelin-1 siRNA가 도입된 RAW-ASC 세포에서는 TNF-α 생성량이 약 5% 증가한 반면, NLRP3 및 ASC의 발현에 대한 7-MSI의 억제 효과는 감소됨을

확인하였다. 이와 같은 결과는 7-MSI가 atuophagy를 활성화시켜 멜라닌 생성을 억제할 뿐만 아니라 inflammasome 형성을 억제하여 염증반응을 저하시킬 수 있음을 시사하는 것이다. 이상의 결과들은 7-MSI가 1) 멜라닌 합성 억제, 2) 전염증성 사이토카인의 발현 및 inflammasome 형성 억제를 통한 염증반응의 감소, 3) MAPK 신호를 통한 autophagy 시스템 활성화를 통해 미백, 피부 노화방지 및 항염증 등의 다양한 효과를 지니고 있음을 시사하는 것이다. 결론적으로 본 연구에서 얻은 결과들은 7-MSI가 다기능 피부 항노화 화장품 소재 및 피부질환 치료제로 개발될 수 있는 가능성을 지니고 있음을 보여준다.

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