



저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:



저작자표시. 귀하는 원저작자를 표시하여야 합니다.



비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 [이용허락규약\(Legal Code\)](#)을 이해하기 쉽게 요약한 것입니다.

[Disclaimer](#)

약학석사학위논문

**Anti-melanogenic Activity of
Demethoxycurcumin via
Microphthalmia-associated
Transcription Factor
(MITF) in Melanocyte**

Demethoxycurcumin의 멜라닌 생성 조절 작용 기전

2016년 2월

서울대학교 대학원
약학과 천연물과학전공
김 지 혜

ABSTRACT

Anti-melanogenic Activity of Demethoxycurcumin via Microphthalmia-associated Transcription Factor (MITF) in Melanocyte

Jihye Kim

Natural Products Science

College of Pharmacy

The Graduate School

Seoul National University

Natural products from plants, fungi, bacteria and other organisms continue to be used in pharmaceutical preparations. The anti-melanogenic activity of demethoxycurcumin (DC), a compound of *C. aromatica*, and its molecular mechanism was investigated in mouse melan-a cells. DC significantly inhibited melanin synthesis and down-regulated the expression of the microphthalmia-associated transcription factor (MITF) which suppresses the expression of tyrosinase, tyrosinase-related protein-1, and -2 (TRP-1, -2). Further analysis revealed that DC suppressed the SRY-related HMG-box 10 (SOX10), critical transcription factors of MITF. DC also effectively inhibited DNA-binding activity

of phosphor-cAMP response element binding protein (p-CREB), an up-stream regulatory factor of MITF. DC exhibit the up-regulation of ERK and AKT activation through mitogen-activated protein kinases (MAP kinase) pathway but there is no effect of phosphorylation of MITF because MITF is already decreased by SOX10 and p-CREB-DNA binding. These findings suggest that DC might serve as a candidate for an effective melanogenic inhibitor in melanocytes via the regulation of MITF signaling pathway.

The anti-inflammatory activity of pinosylvin (PS), a natural stilbenoid from the heartwood of *Pinaceae*, was investigated in human keratinocytes, HaCaT cells. PS inhibited the UV-induced production of prostaglandin E₂ (PGE₂) in HaCaT cells. The suppression of PGE₂ productions by PS was correlated with the down-regulation of mRNA and protein expression of cyclooxygenase-2 (COX-2). Further analysis revealed that PS suppressed the activation of MAP kinases, JNK, c-FOS and c-JUN signaling. These findings suggest that PS might serve as a candidate for a potential UV-induced anti-inflammaging agent via the regulation of MAP kinase signaling pathway.

Keywords: demethoxycurcumin, MITF, melan-a cells, melanogenesis, pinosylvin, PGE₂, HaCaT cells, MAP kinases, inflammation

Student number: 2014-21062

Table of Contents

Abstract	i
Table of Contents	iii
List of Figures	vi
List of Tables.....	viii

Part I. Anti-melanogenic Activity of Demethoxycurcumin via Microphthalmia-associated Transcription Factor (MITF) in Melanocyte

I. Introduction.....	2
II. Materials and Methods	6
A. Materials.....	6
1. Reagents and antibodies	6
2. Compounds	7
3. Cell culture	7
B. Methods	9
1. Melanin content assay	9
2. MTT cell viability assay.....	10
3. Western blotting	10
4. RNA extraction and real-time RT PCR.....	11
5. Nuclear extraction	12

6. p-CREB-DNA binding assay	13
7. Statistical analysis	14
III. Results.....	15
A. Inhibition of melanin synthesis by DC in the mouse melan-a cells	15
B. Effect of DC on the expression of TYR, TRP-1 and TRP-2	18
C. Effects of DC on the expression of MITF protein and gene.....	22
D. Inhibition of DC on the expressions of PAX3 and SOX10, MITF promoter genes.....	24
E. Inhibition of DC on the DNA-binding activity of p-CREB.....	26
F. Effect of DC on ERK, AKT and MITF.....	28
G. Inhibition of melanin synthesis by DC in a reconstituted human skin model (Neoderm®).....	30
IV. Discussion	33

Part II. Inhibitory Effect of Pinosylvlin on UVB-induced Inflammation via MAPK Signaling Pathway in Human Keratinocytes

I. Introduction.....	38
II. Materials and Methods	41
A. Materials.....	41
1. Reagents and antibodies	41
2. Compounds	42

3. Cell culture.....	42
B. Methods	44
1. Cell proliferation assay.....	44
2. Prostaglandin E ₂ assay	44
3. Western blotting	44
4. RNA extraction and real-time RT PCR.....	45
5. Statistical analysis	46
III. Results.....	48
A. Inhibition of prostaglandin E ₂ production by PS in UVB irradiated HaCaT cells	48
B. Suppression of COX-2 protein and mRNA expression by PS in UVB irradiated HaCaT cells.....	51
C. Effects of PS on the expression of MAPK signaling pathway	54
IV. Discussion	56
References	59
Abstract in Korean.....	66

List of Figures

Figure 1. Schematic representation of MITF signaling pathway	5
Figure 2. Chemical structure of demethoxycurcumin (DC).....	8
Figure 3. Effect of DC on melanin content	16
Figure 4. Effect of DC on melan-a cell viability	17
Figure 5. Effect of DC on the expression of melanogenesis-related proteins	19
Figure 6. Effect of DC on the expression of TRP-1, TRP-2 and tyrosinase genes	20
Figure 7. Effects of DC on the expression of MITF	23
Figure 8. Effects of DC on Pax3 and Sox10	25
Figure 9. Effect of DC on the protein level and DNA binding activity of p-CREB	27
Figure 10. Effect of DC on the expression of phosphorylation of AKT, ERK and MITF	29
Figure 11. Effect of DC on melanin content in the reconstituted human skin model	31
Figure 12. Effect of DC on the reconstructed human skin cell viability	32
Figure 13. Schematic representation of plausible mechanisms of action of DC in melan-a cells.....	36
Figure 14. Schematic representation of UV-induced signaling pathway	40
Figure 15. Chemical structure of pinosylvin (PS).....	43
Figure 16. Inhibitory effect of PS on UVB-induced PGE2 production	49
Figure 17. Effect of PS on HaCaT cell viability	50
Figure 18. Effect of PS on COX-2 protein expression	52

Figure 19. Effect of PS on COX-2 mRNA expression	53
Figure 20. Effect of PS on the expression of JNK, c-FOS and c-JUN in UVB- irradiated HaCaT cells.....	55
Figure 21. Schematic representation of plausible mechanisms of action of PS in HaCaT cells	58

List of Tables

Table 1. Sequences of target gene-specific primers used in real-time PCR.....	14
Table 2. Sequences of target gene-specific primers used in real-time PCR.....	47

Part I.

**Anti-melanogenic Activity of
Demethoxycurcumin via Microphthalmia-
associated Transcription Factor
(MITF) in Melanocyte**

I. Introduction

Several environmental factors such as UV lights can induce pigmentation of skin. The pigment, melanin, colors our skin and is the primary cause of skin pigmentation. Melanin is produced by melanocytes found in the basal layer of the epidermis. The progression of melanin formation is called melanogenesis.

Melanogenesis is regulated by three major melanocyte specific enzymes, tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) (Hearing and Tsukamoto, 1991). TYR is the key enzyme associated with the colors of skin, hair, and eyes in animals (Kubo et al, 2000). It catalyzes the oxidation of L-tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA) and leads to DOPA quinone formation which is the first step in melanin synthesis (Shin et al, 1998). TRP-2 allows a quicker conversion of dopachrome to 5, 6-dihydroxyindol-2-carboxylic acid (DHICA) while TRP-1 facilitates the formation of carboxyl group-containing DHICA oxidase eumelanins (Hearing, 2011; Vachtenheim & Borovansky, 2010). TYR is required to synthesize red/yellow pheomelanin and black/brown eumelanin which are found in mammals. TRP-1 and TRP-2 are especially crucial for synthesizing eumelanin (Busca & Ballotti, 2000). Melanogenesis is known for its complex process that is balanced by various signal transduction pathways. Melanogenesis signaling pathway is involved with microphthalmia-associated transcription factor (MITF) which is a key transcription regulator for melanogenesis and the expression of TYR, TRP-1, and TRP-2 (Levy,

Khaled & Fisher, 2006; Villareal et al., 2010). MITF is regulated by various upstream transcription regulators including cAMP response element-binding protein (CREB), paired box 3 (PAX3), SRY-related HMG-box 10 (SOX10), and lymphoid-enhancing factor-1 (LEF-1) (Wan, Hu & He, 2011; Vachtenheim & Borovansky, 2010). CREB is an important promoter for MITF expression (Jung, Hwang & Kim, 2010; Kang, Choi & Choi, 2011) and the phosphorylation of CREB at serine 133 in melanocytes increases MITF expression by binding to the cyclic AMP (cAMP)-responsive element (CRE) in melanocytes (Saha, Singh & Sarkar, 2006; Busca & Ballotti, 2000).

Stimulation of c-Kit receptor tyrosine kinase is known to have a critical role in the development and migration of melanocytes. The c-kit signaling is known to activate the mitogen-activated protein kinase (MAPK), glycogen synthase 3 β (GSK3 β) and phosphatidylinositol 3-kinase (PI3K) pathways (Alexeev & Toon, 2006; Jeon et al., 2009; Larribere et al., 2004; Smalley et al., 2008; Lefevre et al., 2004). Several studies reported that MITF transcription have been effectively modulated by MAPKs, such as extracellular signal-regulated kinase (ERK) and serine/threonine-specific protein kinase (AKT) signaling pathway (Ko et al., 2014). ERK phosphorylates MITF at serine 73, which is also followed by MITF ubiquitination and degradation (Wu, Hemesath & Takemoto, 2000).

Curcuma aromatica has been used for a remedy and a food in Southeast Asia. Curcumin, demethoxycurcumin, bisdemethoxycurcumin, ar-turmerone, aromatic-

turmerone, and beta-turmerone are the major bioactive compounds found in *Curcuma* (Park et al., 2011). In modern pharmacological studies, *Curcuma* constituents, particularly curcumin, have been shown to have anti-inflammatory, anti-cancer (Gescher, 2004), anti-oxidative, chemopreventive and potentially chemotherapeutic properties (Aggarwal & Harikumar, 2009; Hatcher et al., 2008).

Recently, the antimelanogenic effects of partially purified *C. aromatica* and curcumin have been reported. However, the effects of other components of this plant on the melanogenesis signaling pathway have not been investigated. Therefore, in this study we examined the anti-melanogenic activity of demethoxycurcumin on melanogenesis and signaling pathways in mouse melan-a cells.

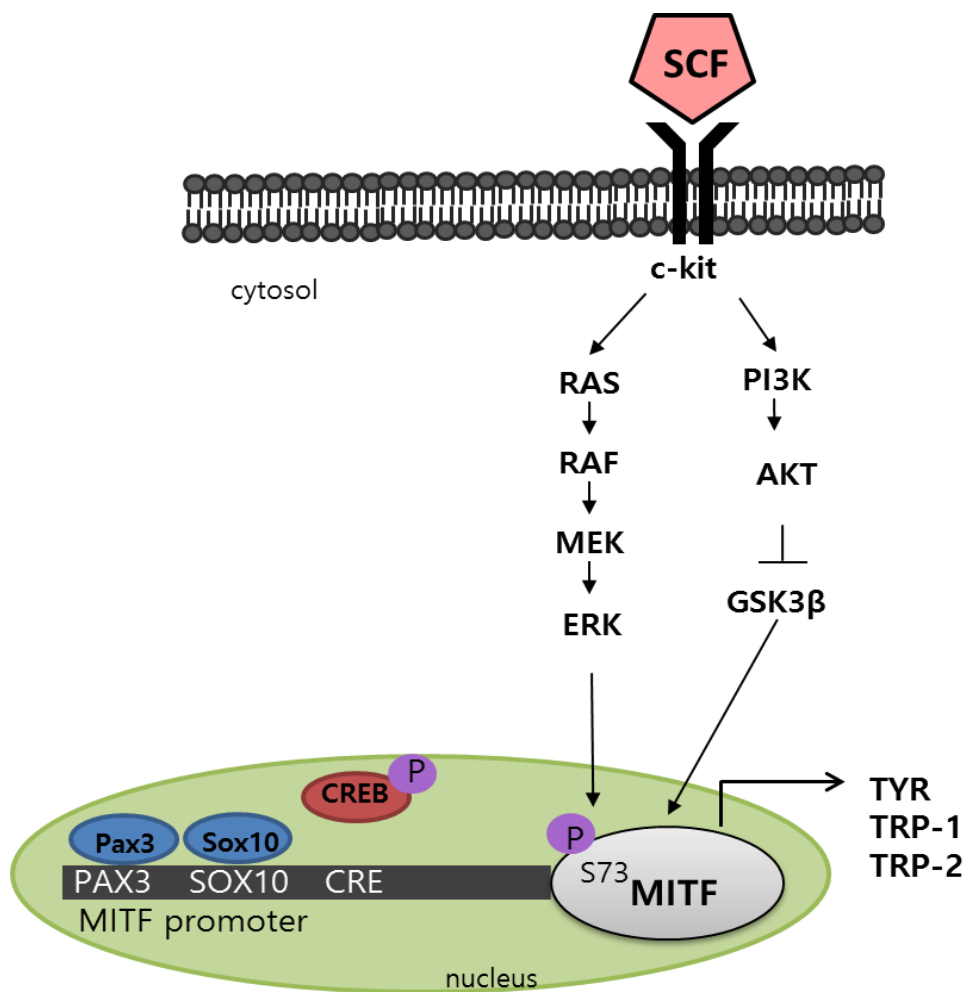


Figure 1. Schematic representation of MITF signaling pathway

II. Materials and Methods

A. Materials

1. Reagents and antibodies

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), antibiotics-antimycotics solution, TRI reagent were purchased from Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and other agents otherwise indicated were purchased from Sigma-Aldrich (St. Louis, CA, USA). Mouse p-GSK3 β (Tyr216) and mouse anti-GSK3 β were purchased from BD Biosciences (San Diego, CA, USA). MITF, tyrosinase, TRP-1, TRP-2, ERK, p-ERK, Pax3, Sox10, β -actin, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, rabbit anti-goat IgG-HRP, rabbit anti-mouse IgG-HRP, mouse anti-goat IgG-HRP, and mouse anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse p-GSK3 β (Ser21/9), c-Kit, AKT, p-AKT (Ser473) and p-CREB were purchased from Cell Signaling Technology (Beverly, MA, USA). p-MITF (Ser73) was purchased from Assay Biotechnology Company (Sunnyvale, CA, USA). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Penzberg, Germany). Gene-specific primers for real-time PCR were synthesized from Bioneer (Daejeon, Korea). TransAM[®] ELISA Kit was purchased from Active Motif Japan (Tokyo, Japan). Neoderm[®]-ME and Neoderm[®]-ED which are the reconstructed human skin models were purchased

from Tego Science (Seoul, Korea)

2. Compound

Demethoxycurcumin (Figure 2) was dissolved in 100% dimethyl sulfoxide (DMSO).

3. Cell Culture

Melan-a cells (originally established by Dr. Bennett at the University of London) were kindly provided by Skin Research Institute, Amore-Pacific Co. (Yongin, Korea). Melan-a cells were grown in RPMI (Rosewell Park Memorial Institute) 1640 medium supplemented with antimyosin (penicillin 100 unit/ml, streptomycin 100 unit/ml, amphotericin B 250 ng/ml), 10% fetal bovine serum (FBS), and 20 nM TPA. The cells were incubated at 37°C in a humidified atmosphere of 10% CO₂. Each experiment was carried out at least three times.

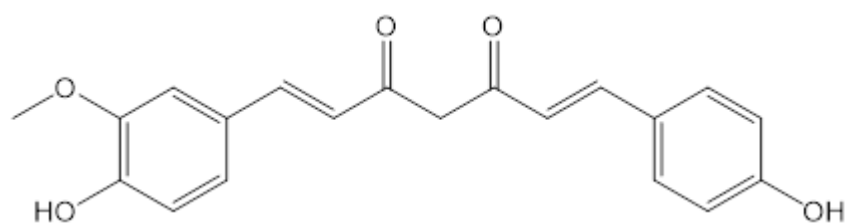


Figure 2. Chemical structure of demethoxycurcumin (DC)

B. Methods

1. Melanin content assay

Confluent cultures of melan-a cells were rinsed with Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS) and lysed with 0.25% trypsin/EDTA. Cells were plated into 6-well plastic culture plates at a density of 4×10^5 cells/well. At 48 h after plating, the media were replaced with test sample. After additional 48 h incubation, the adherent cells exposed to the test samples were assayed. The melanin content was determined as follows: after removing the media and washing the cells with Ca^{2+} and Mg^{2+} -free PBS, the cell pellet was dissolved in 0.1 ml of 1 N NaOH, and incubated at 60°C for 10 min. The optical density at 475 nm was measured by an ELISA reader. For melanin assay using a reconstructed human skin model, Neoderm[®]-ME was purchased and maintained as the manufacturer's instructions (Tego Science, Seoul, Korea). Neoderm[®]-ME was irradiated with 60 mJ/cm² UVB for two times and treated with various concentration of demethoxycurcumin for 4 days. Neoderm[®]-ME was dissolved in 1 N NaOH and the debris was separated by centrifugation at 13, 000 rpm for 15 min. The absorbance at 475 nm was measured from supernatants to determine melanin content.

2. Cell proliferation assay

MTT solution (final concentration of 500 µg/mL) was added to each well and

further incubated for 3 h at 37°C. Each medium was discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve generated formazan. The absorbance was measured at 570 nm, and percent survival was determined by comparison with a control group. For the cell proliferation assay using a reconstructed human skin model, Neoderm[®]-ED was purchased and maintained as the manufacturer's instructions (Tego Science, Seoul, Korea). Neoderm[®]-ED was treated with various concentration of demethoxycurcumin for 3 days. MTT solution (final concentration of 500 µg/mL) was added to each well and further incubated for 3 h at 37°C. Each medium was discarded, Neoderm[®]-ED was dissolved and the debris was separated. The absorbance was measured at 570 nm, and percent survival was determined by comparison with a control group.

3. Western blotting

Melan-a cells were incubated with various concentrations of PS for the indicated times. After incubation, the cells were washed and lysed and the quantitative determination of total protein concentration of each group was done by BCA protein assay. Equivalent levels of protein from each group were subjected to 8-13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with 5 % BSA in a mixture of Tris-buffered saline and Tween 20 (TBST; 1X) for at least 30 min at RT, and then incubated with the desired antibodies

diluted in 2.5% BSA in TBST overnight at 4°C. After washing the membranes three times for 10 min each with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at RT. The membranes were washed three times for 10 min each with TBST, and were then detected with an enhanced chemiluminescence (ECL) detection kit (Intron, Daejeon, Korea). Blots were analyzed by LAS 4000 (GE Healthcare, Little Chalfont, UK).

4. RNA extraction and real-time PCR

Total RNA from cells or tumor tissues was extracted using TRI reagent (Invitrogen) and reverse transcribed using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time PCR was performed using the iQTM SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The thermocycling conditions utilized were 20 sec at 95°C, followed by 40 cycles of 20 sec at 95°C, 20 sec at 56°C, and 30 sec at 72°C, followed by 1 min at 95°C, and 1 min at 55°C. All experiments were performed in triplicate and analysis was performed using the comparative C_T method with β -actin used for normalization. Sequences of primers are listed at Table 3.

5. Nuclear extraction

Nuclear extracts were prepared by the method of Beg et al (Beg et al., 1993).

Briefly, cells were washed in phosphate-buffered saline, pelleted, and resuspended in lysis buffer (10 mM Tris-HCL, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μ M PMSF, and 1.0% NP-40). After 5 min on ice, the lysates were spun at 2500 rpm in a microcentrifuge at 4°C for 4 min. The pelleted nuclei were briefly washed in lysis buffer without NP-40. The nuclear pellet was then resuspended in an equal-volume nuclear extract buffer (20 mM Tris-HCL, pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol). After 10 min incubation at 4°C, the nuclei were briefly vortexed and spun at 14000 rpm for 5 min. The supernatant was then removed and used as a nuclear extract. Protein concentrations were determined by the Bradford assay (Masamoto, Iida & Kubo, 1980).

6. p-CREB-DNA binding assay

p-CREB-DNA binding activity was measured with the Trans AM ELISA kit (Active Motif Japan) was used according to the manufacturer's instructions. Nuclear extracts (2 μ g) were incubated with plate-coated a double-stranded oligonucleotide containing the consensus CRE site (TGACGTCA). Plates were washed, and anti-Ser133-pCREB rabbit antibody was added to the well plates. The binding of antibody was detected with the incubation of an HRP-conjugated secondary antibody and developed with tetramethylbenzidine (TMB) substrate. The reaction intensity was measured by the absorbance at 450 nm.

7. Statistical Analysis

Data were expressed as means $\pm$ standard deviation (SD) for the indicated number of independently performed experiments. Statistical significance was analyzed using the Student's *t*-test or one-way analysis of variance (ANOVA) coupled with the Dunnett's *t*-test. Differences were considered statistically significant at $*P<0.05$ $**P<0.01$, $***P<0.001$.

Table 1. Sequences of target gene-specific primers used in real-time PCR

Target genes		Sequences
Mouse <i>MITF</i>	Sense	5'-CTAAGTGGTCTGCGGTGTCTC-3'
	Antisense	5'-GGTTTTCCAGGTGGGTCTG-3'
Mouse <i>TYROSINASE</i>	Sense	5'-CACCTGAAAATCCTAACTTACTCA-3'
	Antisense	5'-CTCTTCTGATCTGCTACAAATGATCT-3'
Mouse <i>TRP-1</i>	Sense	5'-TGGGAACACTTTGTAACAGCA-3'
	Antisense	5'-ACTGCTGGTCTCCCTACATTTC-3'
Mouse <i>TRP-2</i>	Sense	5'-GGCTACAATTACGCCGTTG-3'
	Antisense	5'-CACTGAGAGAGTTGTGGACCAA-3'
Mouse	Sense	5'-AAGGCCAACCGTGAAAAGAT-3'
<i>β-Actin</i>	Antisense	5'-GTGGTACGACCAGAGGCATAC-3'

III. Results

A. Inhibition of melanin synthesis by DC in the mouse melan-a cells

To determine the effect of demethoxycurcumin (DC) on melanin synthesis, the melanin content was measured. The melan-a cells were treated with various concentrations of DC for 48 h and the results showed that the melanin content of DC treated cells was more significantly decreased in a dose-dependent manner by 35.3%, 56.7% and 66.1% respectively than the control group (Figure 3). These results showed that DC inhibited melanogenesis in melan-a cells.

MTT assay was performed to evaluate the cytotoxicity of DC in melan-a cells. Melan-a cells were treated with various concentrations of DC for 24 h. As shown in Figure 4, the results showed that DC had no cytotoxic effect in melan-a cells.

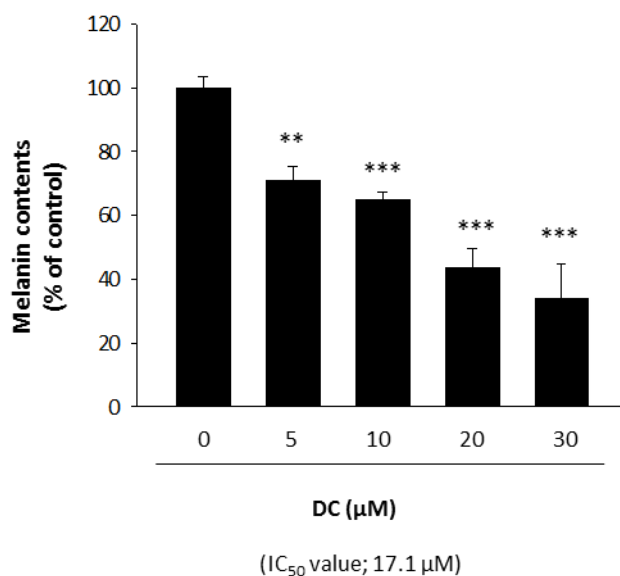


Figure 3. Effect of DC on melanin content

The cells were treated with various concentrations of DC for 48 h. The melanin contents were measured as described the 'Material and Methods'. The results were calculated as the amount of intracellular melanin per cell and expressed as percentage of control (% of control). Data are expressed as mean $\pm$ SD (n=3). * P <0.05 ** P <0.01, *** P <0.001.

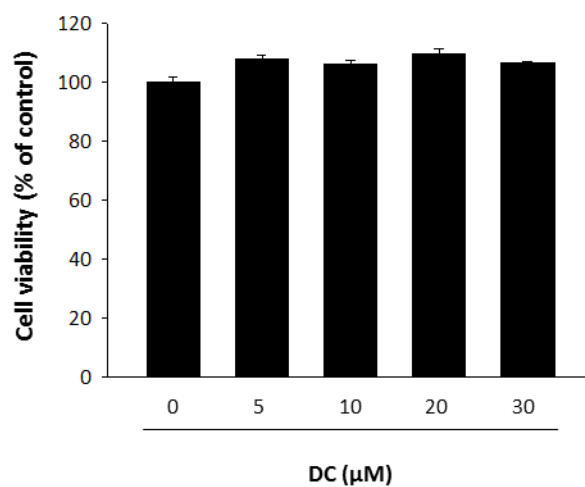


Figure 4. Effect of DC on melan-a cell viability

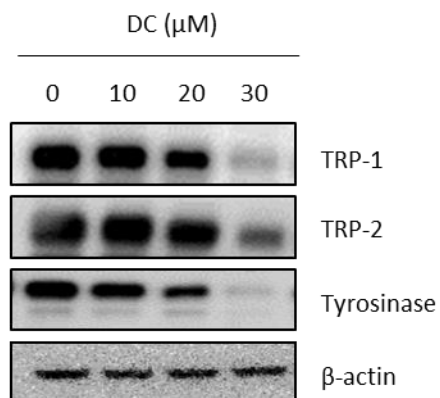
The cells were treated with various concentrations of DC for 24 h. The viability of the cells was determined by a MTT assay. Data are expressed as mean $\pm$ SD (n=3).

B. Effect of DC on the expression of TYR, TRP-1 and TRP-2

To elucidate the mechanism of action mediated by DC (DC) on the inhibition of melanogenesis in melan-a cells, the effects of DC on the expressions of the TYR, TRP-1, and TRP-2 were examined. The cells were treated with DC at 24 and 48 h and then the protein expressions were measured by western blot analysis. As shown in Figure 5, the expression of TYR, TRP-1 and TRP-2 protein expressions were decreased in a concentration-dependent manner at 24 and 48 h.

The mRNA level of *TYR*, *TRP-1* and *TRP-2* were evaluated by RT-PCR analysis. As shown in Figure 6, DC decreased the mRNA levels of *TYR*, *TRP-1* and *TRP-2* in a concentration-dependent manner at 24 h suggesting that DC regulated TYR, TRP-1, and TRP-2 both at the translational protein level and the transcriptional mRNA level.

(A)



(B)

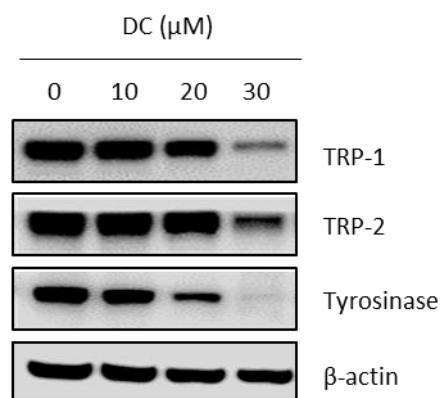


Figure 5. Effect of DC on the expression of melanogenesis-related proteins

The cells were treated with 10, 20 and 30 μ M DC for 24 h (A) and 48 h (B). The expressions of tyrosinase, TRP-1 and TRP-2 proteins were examined by Western blot analysis. β -actin was used as an internal standard.

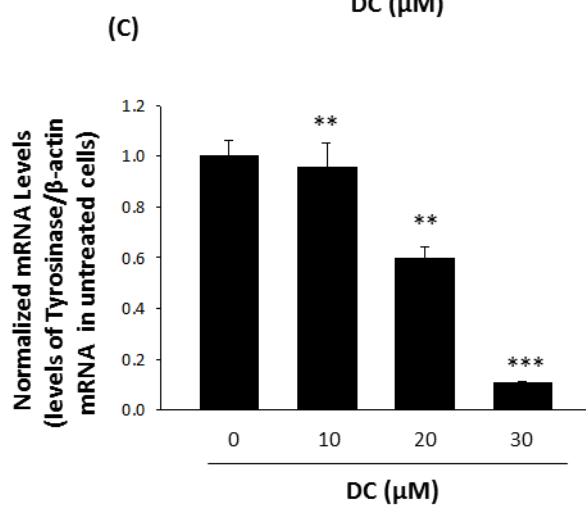
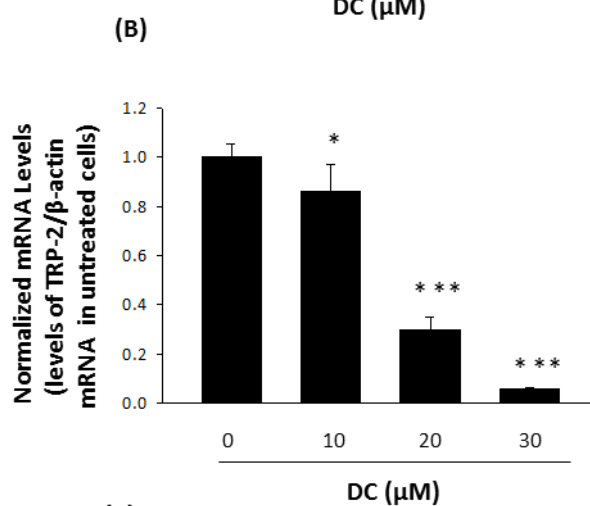
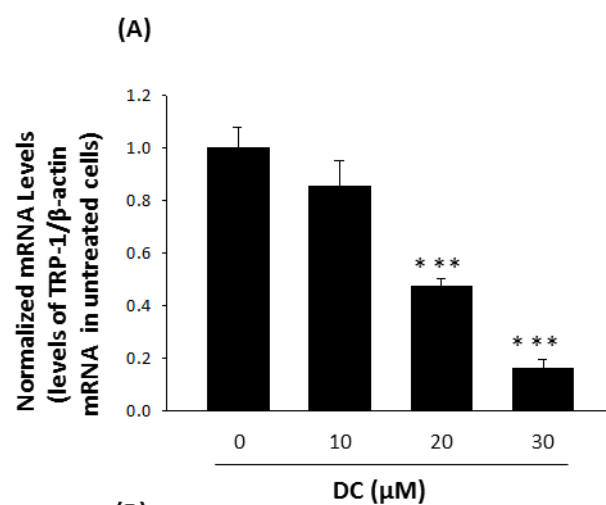


Figure 6. Effects of DC on the expression of *TRP-1*, *TRP-2* and *tyrosinase* genes

The cells were treated with indicated concentrations of DC for 24 h and then the levels of *TRP-1*, *TRP-2* and *tyrosinase* genes were examined by real-time RT-PCR.

Data are expressed as mean $\pm$ SD (n=3). * P <0.05 ** P <0.01, *** P <0.001.

C. Effect of DC on the expression of MITF protein and gene

Microphthalmia-associated transcription factor (MITF) is a transcription factor and it is related in the regulation of melanogenesis. MITF is an important transcriptional regulator of the TRP-1, TRP-2 and tyrosinase. The effect of DC on the expression of *MITF* was investigated by western blot analysis and RT-PCR.

MITF protein and mRNA levels were decreased in dose-dependent manner after 24 h (Figure 7), suggesting that the down-regulation of TRP-1, TRP-2 and tyrosinase might be contributed by the down-regulation of MITF.

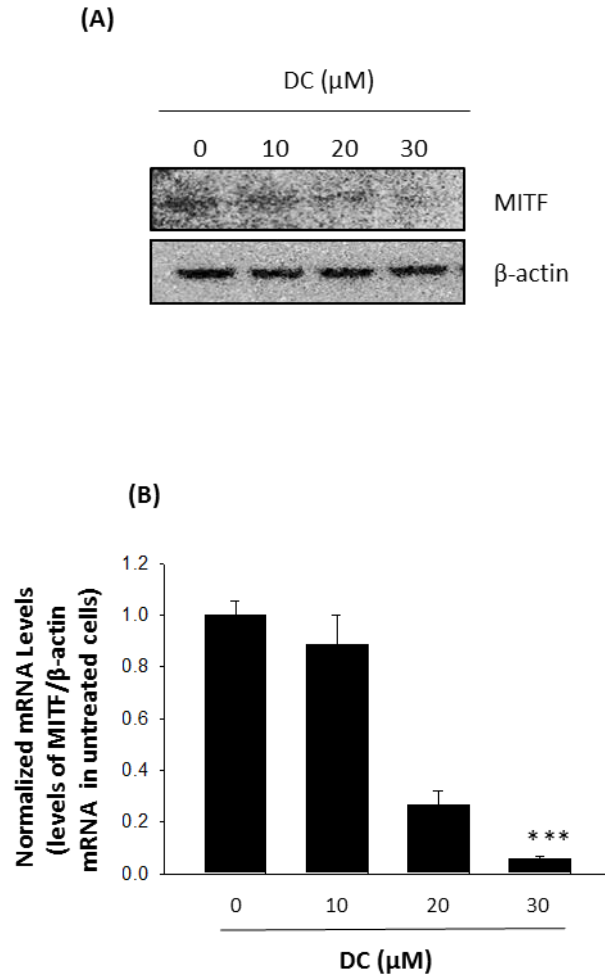


Figure 7. Effect of DC on the expression of *MITF*

The cells were treated with 10, 20 and 30 μM DC for 24 h. The expressions of MITF proteins were examined by Western blot analysis (A). The expressions of *MITF* mRNA were examined by real-time RT-PCR (B). β -actin was used as an internal standard. Data are expressed as mean $\pm$ SD (n=3).

D. Inhibition of DC on the expressions of Pax3 and Sox10, MITF promoter genes

MITF promoter is stimulated by transcription factors, Pax3, Sox10 and CREB. Thus, the effects of DC on these transcription factors were investigated. As shown Figure 8, DC regulated the activation of Sox10 but did not change Pax3 after treating with DC for 24 h. In short time, 15 and 30 min, Sox10 protein expressions were also decreased by 20 μ M of DC. These results suggest that DC regulated Sox10.

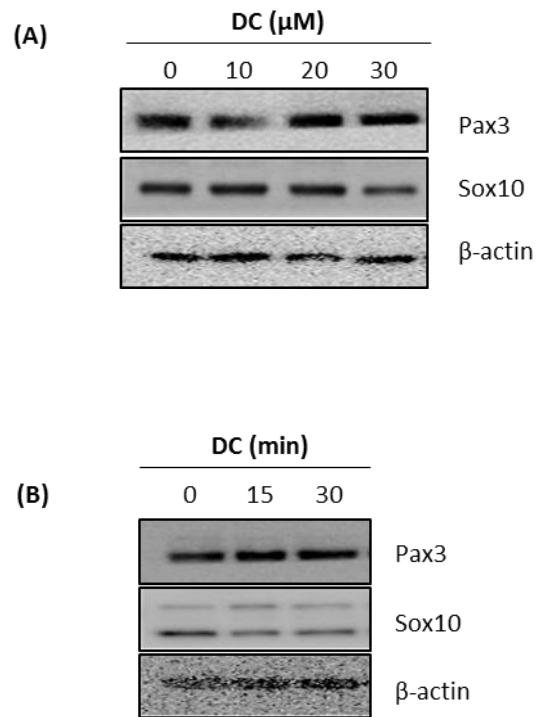


Figure 8. Effects of DC on Pax3 and Sox10

Melan-a cells were treated with indicated concentration of DC for 24 h (A) and treated with 20 μ M of DC for indicated times (B). The expression levels of Pax3 and Sox10 were examined by Western blotting. β -actin was used as an internal standard.

E. Inhibition of DC on the DNA-binding activity of p- CREB

CREB is a crucial transcription factor of MITE. Thus, the effects of DC on the protein level of p-CREB and DNA-binding activity of p- CREB were investigated. As shown Fig.9, the expressions of p-CREB were not changed but the DNA-binding activity of p-CREB was decreased by DC. Using the ELISA-based Trans AM method and specific p-CREB primary Ab, DC significantly inhibited the DNA-binding activity of phosphorylated CREB in a dose-dependent manner. DC was treated for 24 h. This result suggests that DC regulated DNA-binding activity of p-CREB, not reducing protein level.

F. Effect of DC on the expression of ERK, AKT and MITF

DC decreased the protein levels of phosphorylation of ERK and AKT in dose-dependent manner after 24 h. However, the expression of phosphorylation of MITF didn't be changed. This indicated that DC regulated activation of ERK and AKT but not the phosphorylation of MITF (Figure 10). Phosphorylation of MITF didn't be increased because expression of MITF was already decreased by transcription factor of MITF promoter.

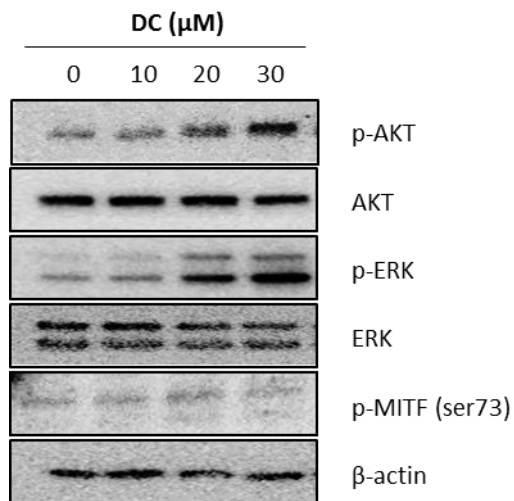


Figure 10. Effects of DC on the expression of phosphorylation of AKT, ERK and MITF

The cells were treated with indicated concentration of DC for 24 h.

G. Inhibition of melanin synthesis by DC in a reconstituted human skin model (Neoderm[®])

To determine the effect of DC on melanin synthesis in human skin, a reconstituted human skin model containing melanocytes (Neoderm[®] -ME) was used. The reconstituted human skin was treated with various concentrations of DC for 4 days and the results showed that the melanin content of DC treated reconstructed human skin was decreased in a dose-dependent manner than the control group (Figure 11). These results imply the possibility of DC as an anti-melanogenic agent which is applicable in human skin.

MTT assay was performed to evaluate the cytotoxicity of DC in a reconstructed human skin containing dermis and epidermis (Neoderm[®] -ED). The reconstructed human skin was treated with various concentrations of DC for 3 days. As shown in Figure 12, the results showed that DC had no toxicity in the reconstructed human skin.

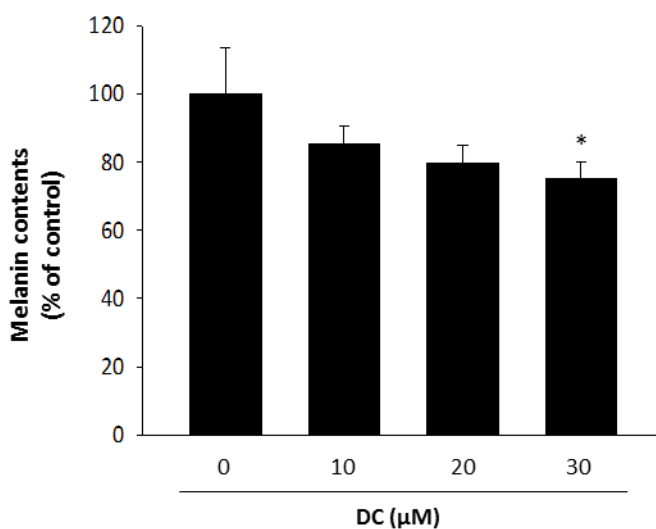


Figure 11. Effect of DC on melanin content in the reconstituted human skin model

The reconstituted human skin model was treated with various concentrations of DC for 4 days. The melanin contents were measured as described the ‘Material and Methods’. The melanin content of the lysates was measured at 475 nm after dissolving the reconstructed human skin samples in 1 N NaOH and the results were expressed as percentage of control (% of control). Data are expressed as mean $\pm$ SD (n=3). * P <0.05 ** P <0.01, *** P <0.001.

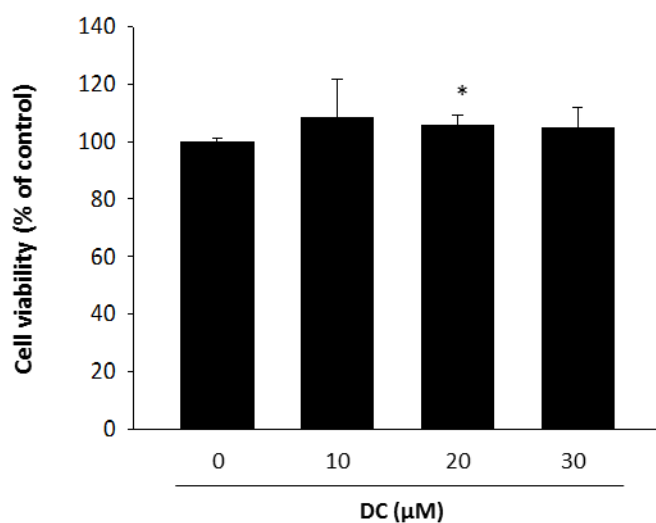


Figure 12. Effect of DC on the reconstructed human skin cell viability

The reconstructed human skin was treated with various concentrations of DC for 3 days. The viability of the cells was determined by a MTT assay. Data are expressed as mean $\pm$ SD (n=3).

IV. Discussion

To our knowledge, this study first demonstrate that DC has an anti-melanogenic effect via regulation of MITF transcription factors in melan-a cells. As confirmed by MTT assay, DC was not toxic at 30 μ M (Figure 4), and the level of melanin under the same concentration was inhibited by 66.1% (Figure 3). To understand the mechanism behind the observed decrease in the melanin content by the DC, the effect of DC on the expressions of the melanogenic enzymes was investigated. TYR, TRP-1 and TRP-2 were evaluated because they are crucial enzyme in melanin synthesis. DC reduced the expression of proteins of TYR, TRP-1 and TRP-2 after the DC treatment (Figure 5). The results showed similar patterns of gene levels which is also decreased after 24 hours (Figure 6). This is clear evidence that DC regulates TYR, TRP-1, and TRP-2 at the transcription level.

MITF is a crucial transcription factor of melanogenesis and regulate the expression of TYR, TRP-1 and TRP-2. MITF protein and gene level was decreased after treatment of DC for 24 h (Figure 7). This is similarly occurred in TYR, TRP-1 and TRP-2, suggesting that the down-regulation of TYR, TRP-1, and TRP-2 by DC can be attributed to the MITF inhibition.

The expression of MITF is regulated by diverse upstream transcription regulators including CREB, Pax3, and SOX10 (Wan, Hu & He, 2011; Vachtenheim & Borovansky, 2010). This showed that DNA binding of p-CREB and Sox10 decreased on DC treated melan-a cells for 24 h (Figure 8, 9).

Stimulation of the c-Kit receptor tyrosine kinase plays a critical role in the development and migration of melanocytes (Yoshida et al., 2001). C-kit signaling controls the MITF expression by the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-e-kinase (PI3K) pathways (Jeon et al., 2009; Larribere et al., 2004; Smalley et al., 2008; Lefevre et al., 2004). To investigate the relation of MAPK pathway with DC, the expression of AKT, ERK and p-MITF (ser73) was examined by 10, 20 and 30 μ M of DC for 24 h. DC increased the protein levels of phosphorylation of ERK and AKT in dose-dependent manner after 24 h. However, the expression of phosphorylation of MITF (ser73) didn't be changed (Figure 10). This result indicated that DC regulated activation of ERK and AKT but not the phosphorylation of MITF. We thought that phosphorylation of MITF didn't be changed because expression of MITF was already decreased by transcription factor of MITF promoter. DC also showed inhibitory effect of melanin synthesis in human skin models without toxicity.

Figure 13 is a schematic representation that summarizes the anti-melanogenic mechanism by DC. The expression level of Sox10 and p-CREB binding activity were suppressed by DC causing that expression of MITF decreased. Inhibited MITF also suppressed the expression of TYR, TRP-1 and TRP-2 causing melanin synthesis decreased. Although DC increases the activation of AKT and ERK, p-MITF is not increased because expression of MITF is already decreased. Conclusively, DC inhibits the melanin production in melanocytes via down-

regulation of MITF, tyrosinase, TRP-1 and TRP-2 expression levels

A plausible mechanism of action for the anti-melanogenic activity of DC via MITF signaling pathway in melan-a cells has been shown for the first time with the suppression of MAPK signaling pathways. These findings suggest that DC can be evaluated further as a whitening agent

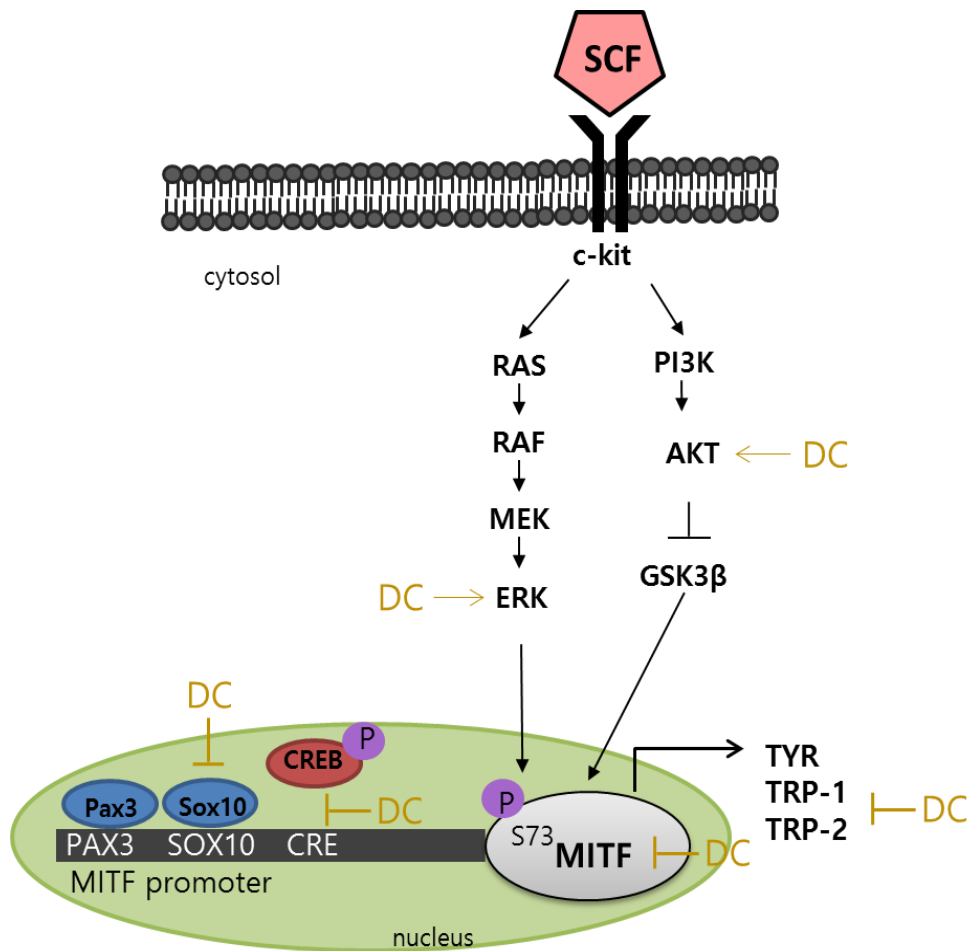


Figure 13. Schematic representation of plausible mechanisms of action of DC in melan-a cells

Part II.
Inhibitory Effect of Pinosylvin on
UVB-induced Inflammation via MAPK
signaling pathway in Human
Keratinocytes

I. Introduction

Many environmental factors such as UV lights and the natural process of aging stimulate the inflammatory process in the skin. UVB penetrates the epidermis and the upper part of the dermis and damages keratinocytes and leads to sunburn, photoaging and skin cancer (Rijken, Kiekens & Bruijnzeel, 2005). UVB radiation promotes the generation of intracellular reactive oxygen species (ROS). ROS play an important role in modulating the inflammatory response and induce many harmful effects (Saito et al, 2004). ROS production after UVB exposure induces mitogen-activated protein kinase (MAPK) cascades such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase, which regulate AP-1 (Yang, Sharrocks & Whitmarsh, 2003). It has been observed that inducible cyclooxygenase (COX-2) is upregulated after UVB exposure in both human and murine skin cells (Chen et al., 2001) and upregulated COX-2 expression in skin epidermis is involved in the development of skin cancer. It leads to increase production of pro-inflammatory mediators such as prostaglandins and thromboxanes (Seo et al., 2002).

Pinosylvin, a natural stilbenoid, is a component of pine leaf (*Pinus densiflora*) and the heartwood of *Pinus* spp (Gehlert, Schoeppner & Kindl, 1990; Bois, Lieutier & Yart, 1999). Resveratrol (3,5,4'-trihydroxy-trans-stilbene), one of the representatives of natural stilbenoid, abundant in grapes, peanuts and pines, has

demonstrated a variety of biological activities such as reduction in the incidence of coronary heart disease, chemoprevention of cancer, growth inhibition of many cancer cells, and anti-inflammatory effects (Surh, 1999; Jang et al., 1997; Pace-Asciak et al., 1995; Jang et al., 1999). Pinosylvin has been shown to have antibacterial, antifungal activities (Lee et al., 2005), antiinflammation (Lee et al., 2006; Park et al., 2004) and antimetastatic activity (Park et al., 2012).

Recently, the antiinflammatory effects of pinosylvin has been reported. However, inhibitory effect of UVB-induced inflammation via the regulation of MAPK signaling pathway by pinosylvin in human keratinocyte, HaCaT cells has not been investigated. Therefore, in this study, the anti-inflammatory effect of pinosylvin via MAPK signaling pathway in human keratinocyte, HaCaT cells.

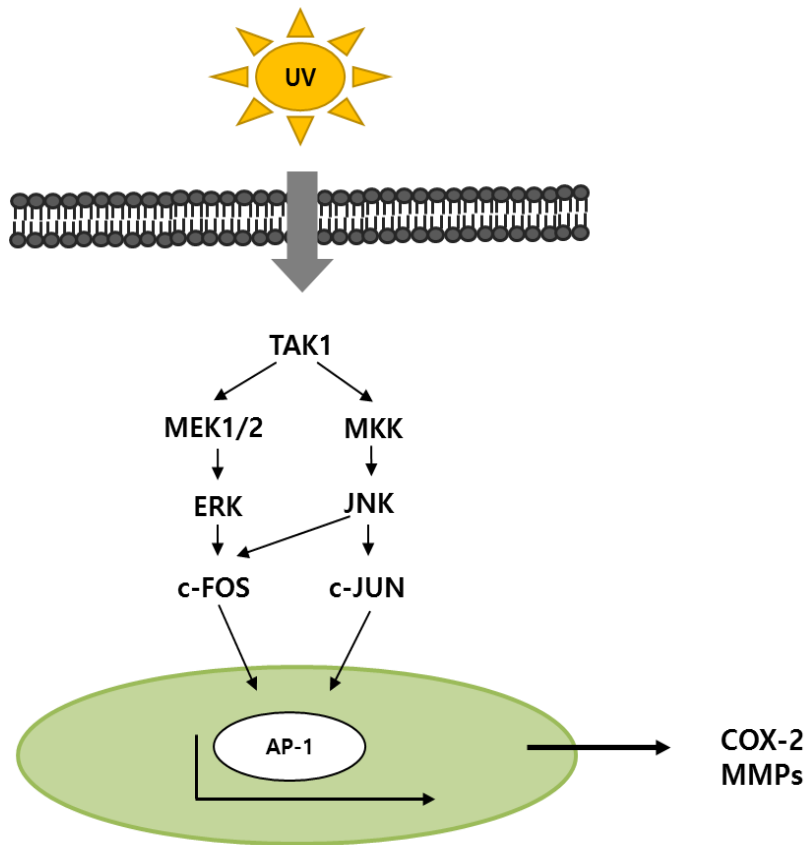


Figure 14. Schematic representation of UV-induced signaling pathway

II. Materials and Methods

A. Materials

1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotics-antimycotics solution, and trypsin-EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, goat anti-goat IgG-HRP, COX-2, c-FOS, c-JUN, ERK1/2 and p-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against SAPK/JNK, p-SAPK/JNK (Thr183/Tyr185), p-c-JUN (Ser73) and p-c-JUN (Ser63) were purchased from Cell Signaling Technology (Beverly, MA, USA). Gene-specific primers were synthesized by Bioneer (Daejeon, Korea). AMV reverse transcriptase, dNTP mixture, random primer, RNasin and Taq polymerase were purchased from Promega (Madison, WI, USA). Enzyme immunoassay kits used for the measurement of PGE₂ was purchased from R&D systems (Minneapolis, USA).

2. Coumpound

Pinosylvin (Figure 15) was dissolved in 100% dimethyl sulfoxide (DMSO).

3. Cell culture

Human keratinocyte, HaCaT cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. Cells was incubated at 37°C, with 5% CO₂ in a humidified atmosphere.

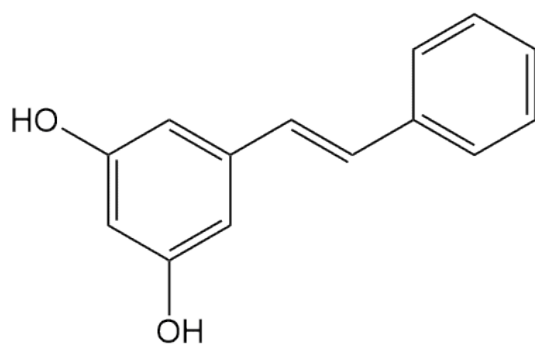


Figure 15. Chemical structure of pinosylvin (PS)

B. Methods

1. Cell proliferation assay

MTT solution (final concentration of 500 $\mu\text{g/mL}$) was added to each well and further incubated for 3 h at 37°C. Each medium was discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve generated formazan. The absorbance was measured at 570 nm, and percent survival was determined by comparison with a control group.

2. Prostaglandin E₂ assay

The expression level of PGE₂ was measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, HaCaT cells (2.5×10^5 cells/mL) were plated and pretreated with the indicated concentration of pinosylvin for 2 h prior to stimulation with 15 mJ/cm² of UVB for 18 h. Culture medium supernatants were collected for the determination of PGE₂ concentration by ELISA.

3. Western blotting

HaCaT cells were incubated with various concentrations of pinosylvin for the indicated times. After incubation, the cells were washed and lysed and the quantitative determination of total protein concentration of each group was done by BCA protein assay. Equivalent levels of protein from each group were subjected to

8-13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with 5 % BSA in a mixture of Tris-buffered saline and Tween 20 (TBST; 1X) for at least 30 min at RT, and then incubated with the desired antibodies diluted in 2.5% BSA in TBST overnight at 4°C. After washing the membranes three times for 10 min each with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at RT. The membranes were washed three times for 10 min each with TBST, and were then detected with an enhanced chemiluminescence (ECL) detection kit (Intron, Daejeon, Korea). Blots were analyzed by LAS 4000 (GE Healthcare, Little Chalfont, UK).

4. RNA extraction and real-time PCR

Total RNA from cells or tumor tissues was extracted using TRI reagent (Invitrogen) and reverse transcribed using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time PCR was performed using the iQTM SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The thermocycling conditions utilized were 20 sec at 95°C, followed by 40 cycles of 20 sec at 95°C, 20 sec at 56°C, and 30 sec at 72°C, followed by 1 min at 95°C, and 1 min at 55°C. All experiments were performed in triplicate and analysis was performed using the comparative C_T

method with β -actin used for normalization. Sequences of primers are listed at Table 3.

5. Statistical Analysis

Data were expressed as means $\pm$ standard deviation (SD) for the indicated number of independently performed experiments. Statistical significance was analyzed using the Student's *t*-test or one-way analysis of variance (ANOVA) coupled with the Dunnett's *t*-test. Differences were considered statistically significant at * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$.

Table 2. Sequences of target gene-specific primers used in RT-PCR

Target genes		Sequences
Human	Sense	5' – CTTCACGCATCAGTTTTTCAAG – 3'
<i>COX-2</i>	Antisense	5' – TCACCGTAAATATGATTTAAGTCCAC – 3'
Human	Sense	5' – AGCACAATGAAGATCAAGAT – 3'
<i>β-actin</i>	Antisense	5' – TGTAACGCAACTAAGTCATA – 3'

III. Results

A. Inhibition of prostaglandin E₂ production by PS in UVB irradiated HaCaT cells

To determine the inhibitory effect of pinosylvin (PS) on prostaglandin E₂ (PGE₂) production, a PGE₂ enzyme immunometric assay (EIA) was used. The irradiation of keratinocyte cells with 15 mJ/cm² of UVB for 18 h dramatically increased the production of PGE₂ from the basal level of 18 pg/mL without UVB to 147 pg/mL. When the cells were treated with PS for 18 h, the PGE₂ production induced by UVB was significantly inhibited in a concentration-dependent manner (Figure 16). Indomethacin, a positive control, resulted in 18 pg/mL PGE₂ under the same assay conditions (data not shown).

MTT assay was performed to evaluate the cytotoxicity of PS in HaCaT cells after 15 mJ/cm² of UVB irradiation. No significant effect on cell viability was observed at test concentrations of PS, as determined by the MTT assay, indicating that the inhibition of PGE₂ production by PS was not mediated by a cytotoxic effect (Figure 17).

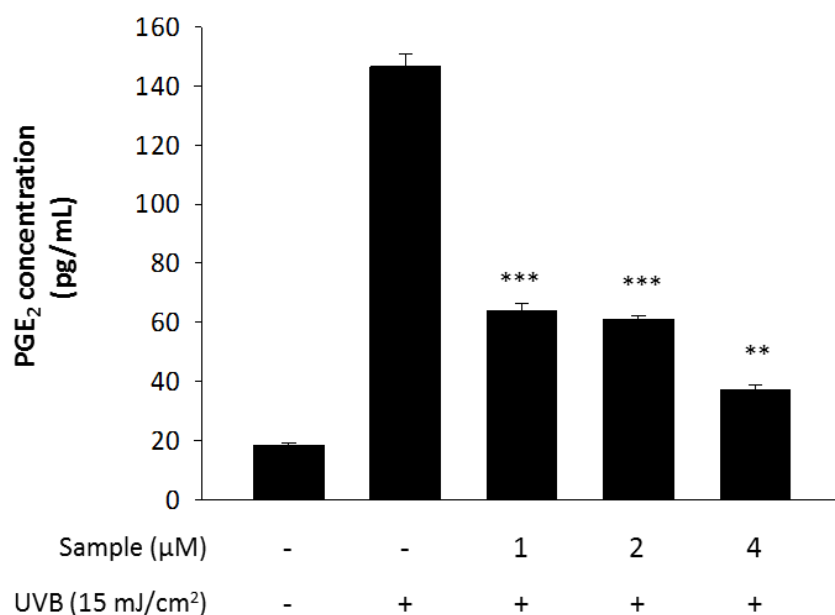


Figure 16. Inhibitory effect of PS on UVB induced PGE₂ production

HaCaT cells were irradiated with UVB (15 mJ/cm^2) in the presence or absence of PS. After incubation for 18 h, the amount of PGE₂ in the supernatants was determined by enzyme immunoassay. Data are expressed as mean $\pm$ SD (n=3).

*P<0.05 **P<0.01, ***P<0.001.

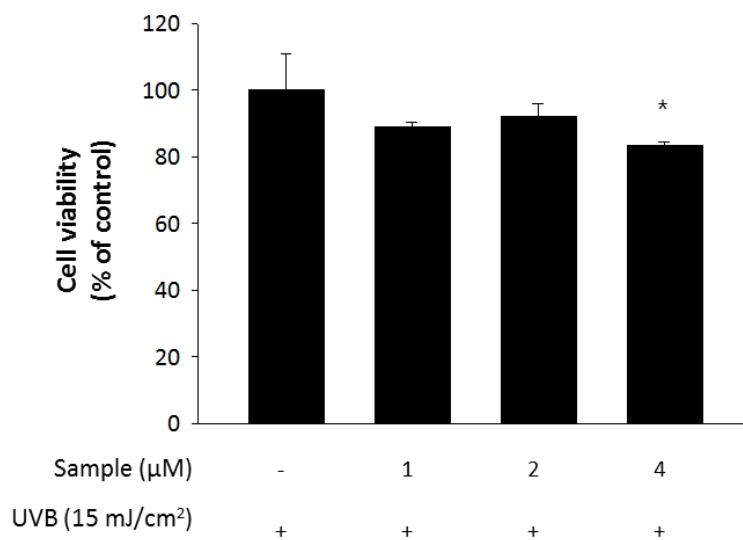


Figure 17. Effect of PS on HaCaT cell viability

The cells were treated with various concentrations of PS for 24 h. The viability of the cells was determined by MTT assay. Data are expressed as mean $\pm$ SD (n=3).

*P<0.05 **P<0.01, ***P<0.001.

B. Suppression of COX-2 protein and mRNA expression by PS in UVB irradiated HaCaT cells

The overproduction of PGE₂ is associated with the overexpression of COX-2. To elucidate the mechanism of action mediated by PS on the inhibition of PGE₂ production, the effects of PS on COX-2 protein and gene expression were determined. HaCaT cells were incubated after UVB (15 mJ/cm²) irradiation in the presence or absence of various concentrations of PS. After 18 h incubation, total mRNA and protein were isolated and further analyzed by RT-PCR and Western blotting, respectively. PS suppressed the UV-induced overexpression of COX-2 protein levels in a concentration-dependent manner (Figure 18).

To further investigate the effect of PS on the UV-induced enhancement of mRNA expression, steady-state levels of *COX-2* mRNA were evaluated by RT-PCR analysis. PS suppressed the UV-induced overexpression of *COX-2* mRNA levels, suggesting that the inhibition of PGE₂ production by PS might be correlated with the suppression of COX-2 expression at the translational and transcriptional levels (Figure 19).

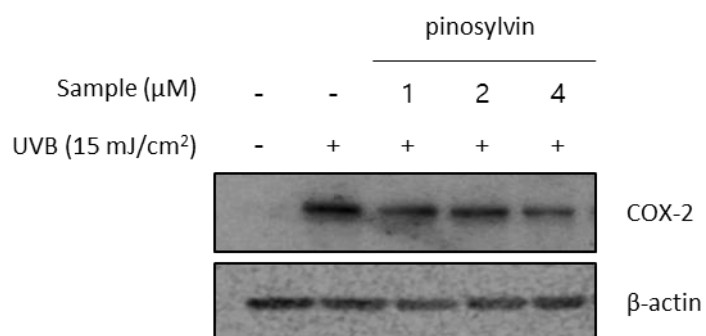


Figure 18. Effect of PS on COX-2 protein expression

HaCaT cells were irradiated by 15 mJ/cm² of UVB with or without various concentrations of PS for 24 h. Total protein were isolated and further analyzed by western blotting.

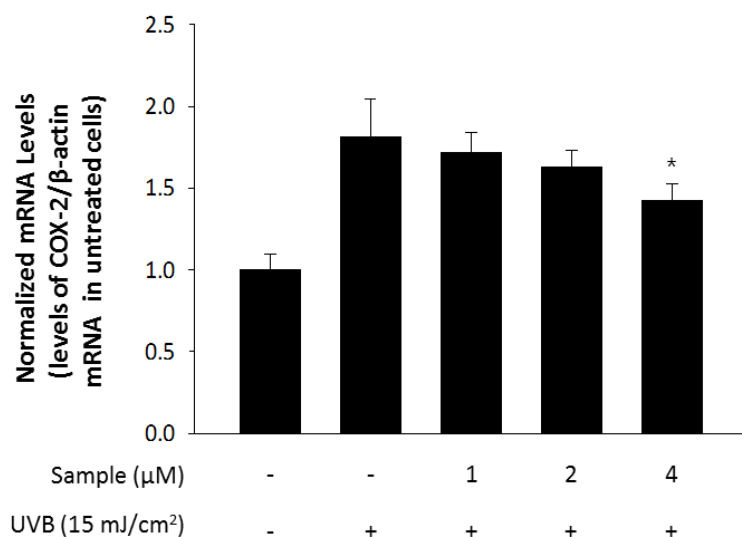


Figure 19. Effect of PS on COX-2 mRNA expression

HaCaT cells (2.5×10^5 cells/mL) were irradiated with 15 mJ/cm^2 of UVB with or without various concentrations of PS for 24 h. The expressions of COX-2 mRNA were examined by real-time RT-PCR. Data are expressed as mean $\pm$ SD (n=3).

*P<0.05 **P<0.01, ***P<0.001.

C. Effect of PS on the expression of MAPK signaling pathway

UV lights activate MAPK family members, such as JNK and ERK. The MAPK signaling pathway plays a crucial role in mediating the induction of pro-inflammatory cytokines (Christman, Lancaster & Blackwell, 1998; Guha, 2001). PS suppressed the activation of JNK in UVB-induced HaCaT. The down-stream of JNK also was investigated by western blotting. The expression of c-FOS and c-JUN was suppressed by PS in UVB-induced HaCaT cells (Figure 20). These data suggest that the anti-inflammatory activity of PS was also partly associated with the suppression of MAPK signaling pathway.

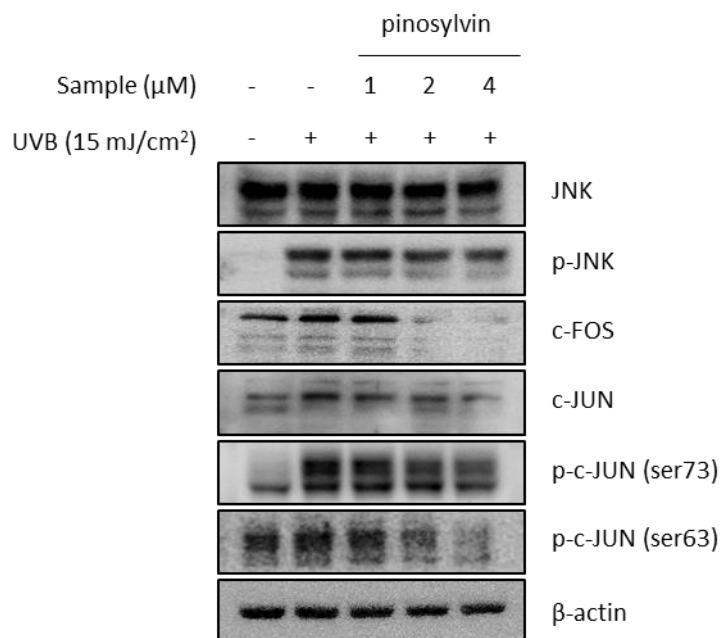


Figure 20. Effect of PS on the expression of JNK, c-FOS and c-JUN in UVB-irradiated HaCaT cells

HaCaT cells (2.5×10^5 cells/mL) were treated with various concentrations of PS for 24 h prior to UVB irradiation. After UVB irradiation for an additional 30 min, proteins were extracted and analyzed by western blotting.

IV. Discussion

In the course of searching bioactive compounds from natural products with the inhibition of UV-induced inflammation, PS inhibited the production of PGE₂ in UVB-induced HaCaT cells. These inhibitory effects were accompanied by decreasing in the expression of COX-2 protein and mRNA, indicating that the impaired release of PGE₂ can be attributed to the inhibition of COX-2 expression at the transcriptional and translational levels.

It is also known that the acute inflammation and development of skin cancer through increased expression of COX-2 are correlated with activation of ERK, JNK and p38 MAPK kinase pathway (Chen et al., 2001; Lin et al., 2004; Mahns et al., 2004). UVB induces the expression of COX-2 by activation of p38 MAPK in HaCaT cells (Chen et al., 2001) or by activation of p38 MAPK and JNK in artificial epidermis (Mahns et al., 2004). In this study, the results showed that JNK signaling was increased by UVB irradiation and attenuated by PS in HaCaT cells. PS inhibited UVB-induced c-Jun phosphorylation and c-Fos expression which is down-stream of activated JNK. These results indicate that PS reduce UVB-induced c-Jun and c-Fos expression by inhibiting phosphorylation of JNK. PS effectively suppresses UVB-induced activation of JNK as well as expression of COX-2, thus suggesting that PS may exert its inhibitory effect on UVB-induced COX-2 expression by inhibiting JNK activities.

In summary, this study demonstrates the potent anti-inflammatory activity of PS in UVB-induced acute inflammation. A plausible mechanism of action for the anti-inflammatory activity of PS has been shown for the first time with the suppression of JNK signaling pathways. These findings suggest that PS is a promising candidate for the management of UV-induced inflammatory responses in human skin.

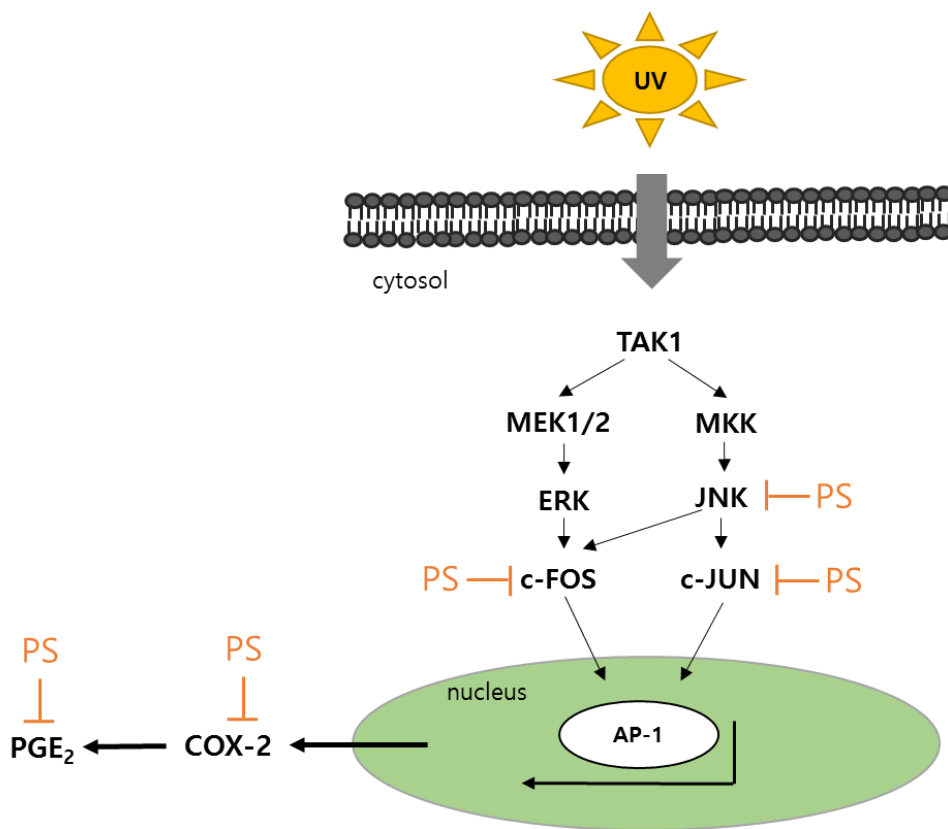


Figure 21. Schematic representation of plausible mechanisms of action of PS in HaCaT cells

References

- Aggarwal BB, Harikumar KB. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int J Biochem Cell Biol* 2009;41:40–59.
- Alexeev V, Toon K. Distinctive role of the c-Kit receptor tyrosine kinase signaling in mammalian melanocytes. *J Invest Dermatol* 2006;126:1102-1110.
- Beg AA, Finco TS, Nanterment PV, Baldwin AS. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol* 1993;13, 3301-3310.
- Bellei B, Flori E, Izzo E, Maresca V, Picardo M. GSK3beta inhibition promotes melanogenesis in mouse B16 melanoma cells and normal human melanocytes. *Cell signal* 2008;20: 1750-1761.
- Bois E, Lieutier F, Yart A. Bioassays on *Leptographium wingfieldii*, a Bark Beetle Associated Fungus, with Phenolic Compounds of Scots Pine Phloem. *Eur J Plant Pathol*. 1999;105:51.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72: 248-254.
- Busca R, Ballotti R. Cyclic AMP a Key Messenger in the Regulation of Skin Pigmentation. *Pigment Cell Res* 2000;13: 60-69.
- Chen W, Tang Q, Gonzales MS, Bowden GT. Role of p38 MAP kinases and ERK

- in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. *Oncogene* 2001;20:3921-3926.
- Christman JW, Lancaster LH, Blackwell TS. Nuclear factor κ B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Med.* 1998;24:1131-1138.
- Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell signal* 2005;17: 1343-1351.
- Ekholm SV, Reed SI. Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. *Curr Opin Cell Biol* 2000;12: 676-684.
- Gehlert R, Schoeppner A, Kindl H. Stilbene synthase from seedlings of *Pinus sylvestris*: Purification and induction in response to fungal infection. *Mol Plant-Microb Interact.* 1990;3:444.
- Gescher A. Polyphenolic phytochemicals versus nonsteroidal anti-inflammatory drugs: which are better cancer chemopreventive agents? *J Chemother* 2004;16 (Suppl 4):3–6.
- Granter SR, Weilbaecher KN, Quigley C, Risher DE. Role for microphthalmia transcription factor in the diagnosis of metastatic malignant melanoma. *Appl Immunohistochem Mol Morphol* 2002;10: 47-51.
- Guha M, O'Connell MA, Pawlinski R, Hollis A, McGovern P, Yan SF, Stern D, Mackman N. Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor α expression by inducing Elk-1 phosphorylation and Egr-1 expression. *Blood*

- 2001;98:1429-1439.
- Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. *Cell Mol Life Sci* 2008;65:1631–1652.
- Hearing VJ. Determination of Melanin Synthetic Pathways. *J Invest Dermatol* 2011;131:E8-E11.
- Hearing VJ, Tsukamoto K. Enzymatic control of pigmentation in mammals. *FASEB J* 1991;5: 2902-2909.
- Imokawa G, Yada Y, Morisaki N, Kimura M. Signaling mechanisms of endothelin-induced mitogenesis and melanogenesis in human melanocytes. *Biochem J* 1996;330: 1235-1239.
- Jang DS, Kang BS, Ryu SY, Chang IM, Min KR, Kim Y. Inhibitory effects of resveratrol analogs on unopsonized zymosan-induced oxygen radical production. *Biochem. Pharmacol.*, 1999;57:705–712.
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HSS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. *Science*. 1997;275:218-220.
- Jeon S, Kim NH, Kim JY, Lee AY. Stem cell factor induces ERM proteins phosphorylation through PI3K activation to mediate melanocyte proliferation and migration. *Pigment Cell Melanoma Res* 2009;22: 77-85.
- Jung E, Hwang W, Kim S. Depigmenting action of platycodin D depends on the cAMP/Rho-dependent signalling pathway. *Exp Dermatol* 2010;20: 986-991.
- Kang YG, Choi EJ, Choi Y. 5,7-Dimethoxyflavone induces melanogenesis in

- B16F10 melanoma cells through cAMP-dependent signalling. *Exp Dermatol* 2011;20: 445-447.
- Ko HH, Chiang YC, Tsai MH, Liang CJ, Hsu LF, Li SY, Wang MC, Yen FL, Lee CW. Eupafolin, a skin whitening flavonoid isolated from *Phyllanthus nodiflorus*, downregulated melanogenesis: Role of MAPK and Akt pathways. *Journal of Ethnopharmacology* 2014;151(1): 386-393.
- Kubo I, Kinst-Hori I, Chaudhuri SK, Kubo Y, Sanchez Y, Ogura T. Flavonols from *Heterotheca inuloides*: tyrosinase inhibitory activity and structural criteria. *Bioorg Med Chem* 2000;8: 1749-1755.
- Larribere L, Khaled M, Tartare-Deckert S, Busca R, Luciano F, Bille K. PI3K mediates protection against TRAIL-induced apoptosis in primary human melanocytes. *Cell Death Differ* 2004;11: 1084-1091.
- Lee SK, Lee HJ, Min HY, Park EJ, Lee KM, Ahn YH, Cho YJ, Pyee JH. Antibacterial and antifungal activity of pinosylvin, a constituent of pine. *Fitoterapia* 2005;76:258-260.
- Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z. Roles of stem cell factor/c-Kit and effects of Gilvec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem* 2004;279: 31769-31779.
- Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med* 2006;12: 406-414.
- Masamoto T, Iida S, Kubo M. Inhibitory effect of Chinese crude drugs on tyrosinase. *Planta Med* 1980;40: 361-365.
- Pace-Asciak CR, Hahn S, Diamndis EP, Soleas G, Goldberg DM. The red wine

- phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implications for protection against coronary heart disease *Clin. Chim. Acta.* 1995;235:207–219.
- Park SY, Jin ML, Kim YH, Kim YH, Lee SJ. Aromatic-turmerone inhibits α -MSH and IBMX-induced melanogenesis by inactivating CREB and MITF signaling pathways. *Arch Dermatol Res* 2011; 303:737–744.
- Park EJ, Min HY, Ahn YH, Bae CM, Pyee JH, Lee SK. Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E₂ production in lipopolysaccharide-induced mouse macrophage cells. *Bioorganic & Medicinal Chemistry Letters*. 2004;14:5895–5898.
- Pillai S, Oresajo C, Hayward J. Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation-a review. *Int J Cosmetic Sci* 2005;27:17-34.
- Rijken F, Kiekens RC, Bruijnzeel PL. Skin-infiltrating neutrophils following exposure to solar-simulated radiation could play an important role in photoageing of human skin. *Br. J. Dermatol* 2005;152:321-328.
- Saha B, Singh SK, Sarkar C. Activation of the Mitf promoter by lipid-stimulated activation of p38-stress signalling to CREB. *Pigment Cell Res* 2006;19: 595-605.
- Saito Y, Shiga A, Yoshida Y, Furuhashi T, Fujita Y, Niki E, Effects of a novel gaseous antioxidative system containing a rosemary extract on the oxidation induced by nitrogen dioxide and ultraviolet radiation. *Biosci.*,

- Biotechnol., Biochem. 2004;68:781-786.
- Seo SJ, Choi HG, Chung HJ and Hong CK. Time course of expression of mRNA of inducible nitric oxide synthase and generation of nitric oxide by UVB in keratinocytes cell lines. *Br. J. Dermatol.* 2002;147:655-662.
- Shin NH, Ryu SY, Choi EJ, Kang SH, Chang IM, Min KR, Kim Y. Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochem Biophys Res Commun* 1998;243: 801-803.
- Shaywitz AJ, Greenberg ME. CREB: A stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 1999;68: 821-861.
- Smalley KS, Contractor R, Nguyen TK, Xiao M, Edwards R, Muthusamy V. Identification of a novel subgroup of melanomas with KIT/cyclin-dependent kinase-4 overexpression. *Cancer Res* 2008;68: 5743-5752.
- Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutat. Res.* 1999;428:305–327
- Vachtenheim J, Borovansky J. Lighting a path to pigmentation: mechanisms of MITF induction by UV. *Exp Dermatol* 2010;19:617-627.
- Vachtenheim J, Borovansky J. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. *Exp Dermatol* 2010;19: 617-627.
- Villareal MO, Han J, Tamada P, Shingemori H, Isoda H. Hirseins inhibit melanogenesis by regulating the gene expressions of Mitf and melanogenesis enzymes. *Exp Dermatol* 2010;19: 450-457.
- Wan P, Hu Y, He L. Regulation of melanocyte pivotal transcription factor MITF by

- some other transcription factors. *Mol Cell Biochem* 2011;354: 241-246.
- Wu M, Hemesath TJ, Takemoto CM. *Genes Dev* 2000;14: 301-312.
- Xu Y, Fisher GJ. Ultraviolet (UV) light irradiation induced signal transduction in skin photoaging. *J. Dermatol. Sci., Suppl.* 2005;1:S1-S8.
- Yang SH, Sharrocks AD, Whitmarsh AJ. Transcriptional regulation by the MAP kinase signaling cascades. *Gene* 2003;320:3-21.
- Yoshida H, Kunisada T, Grimm T, Nishimura EK, Nishioka E, Nishikawa SI. Review: melanocyte migration and survival controlled by SCF/c-kit expression. *J Investig Dermatol Symp Proc* 2001;6: 1-5.

국문초록

Demethoxycurcumin의 멜라닌 생성 조절 작용 기전 연구

서울대학교 대학원 약학과

천연물과학 전공

김지혜

사람의 피부색을 결정하는 주요 요인인 멜라닌은 표피의 기저층에 존재하는 melanocytes에서 여러 단계에 걸쳐 합성되며 이 모든 과정을 melanogenesis 라고 한다. 본 연구의 대상 물질인 demethoxycurcumin (DC)은 강황에 함유된 천연물질로 항암, 염증, 알츠하이머에 대한 치료 효과가 보고 되어 있지만, 미백 효능에 대한 기전 연구는 보고된 바 없다. 따라서 본 연구에서는 DC의 tyrosinase 및 멜라닌 생성 억제효능을 분석하여 그 작용기전에 대해 밝히고자 하였다. 그 결과 DC는 tyrosinase 활성과 melanocyte에서의 멜라닌 생성을 억제하였다. 또한 melanogenesis 관련 효소계인 tyrosinase, tyrosinase-related protein-1 과 -2 (TRP-1, -2)의 단백질 및 유전자 발현을 효과적으로 억제하였다. Microphthalmia-associated transcription factor (MITF)는 멜라닌 생성 시 중요한 역할을 하는 전사인자로 DC의 MITF에 대한 저해 활성을 확인한 결과 단백질 및 유전

자 발현이 감소하였다. MITF의 중요한 전사 인자인 SRY-related HMG-box 10 (SOX10)의 단백질 발현을 억제하고, phospho cAMP response element binding protein (p-CREB)의 DNA binding 활성을 억제함으로써 MITF의 유전자 발현을 저해시킨다는 것을 확인하였다.

MITF의 상위 조절 인자의 단백질 발현을 분석한 결과 DC에 의하여 직접적으로 phospho extracellular signal-regulated kinase (p-ERK), phospho serine/threonine-specific protein kinase (p-AKT) 단백질 발현이 증가하였지만 phospho MITF (ser73)은 증가하지 않았다. 이는 앞서 MITF의 발현량 자체의 감소로 인하여 phospho MITF (ser73)의 발현에는 영향이 미미하였을 것으로 여겨진다. 또한 DC는 3D 인공피부 모델에서도 멜라닌 합성 저해 효능을 보였다. 본 연구를 통하여 DC는 melanocytes에서 MITF를 조절하여 melanogenesis를 억제하는 유용한 미백 후보 물질이 될 것으로 사료된다.

피부 노화의 가장 주된 원인 중 하나인 자외선에 오래 노출되면 염증 반응, 피부암, 조기 노화를 유발한다. Pinosylvin (PS)은 소나무에 함유된 stilbenoid 계열의 성분이다. PS는 암예방효과 및 전이억제 작용, 항진균 등의 효능들이 보고된 바 있다. 본 연구에서는 PS가 자외선에 의해 유도되는 염증반응을 억제할 수 있는지를 알아보았다. PS는 HaCaT cells에서 UVB에 의해 유도되는 prostaglandin E₂ (PGE₂) 생성을 저해하는 효능을 보였으며 cyclooxygenase-2 (COX-2)의 단백질 발현을 억제하였다. 본 연구를 통하여 PS는 keratinocytes에서 자외선에 의해 유도되는 염증반응을 억제하는 유용한 후보 물질이 될 것으로 사료된다.

주요어: demethoxycurcumin, MITF, melan-a cells, melanogenesis,
pinosylvin, PGE₂, HaCaT cells, MAP kinases, inflammation
학번: 2014-21062