

Diphlorethohydroxycarmalol Suppresses Ultraviolet B-Induced Matrix Metalloproteinases via Inhibition of JNK and ERK Signaling in Human Keratinocytes

Mei Jing Piao¹, Madduma Hewage Susara Ruwan Kumara¹, Ki Cheon Kim¹, Kyoung Ah Kang¹, Hee Kyoung Kang¹, Nam Ho Lee² and Jin Won Hyun^{1,*}

¹School of Medicine, ²Department of Chemistry, College of Natural Sciences, Jeju National University, Jeju 63243, Republic of Korea

Abstract

Skin aging is the most readily observable process involved in human aging. Ultraviolet B (UVB) radiation causes photo-oxidation via generation of reactive oxygen species (ROS), thereby damaging the nucleus and cytoplasm of skin cells and ultimately leading to cell death. Recent studies have shown that high levels of solar UVB irradiation induce the synthesis of matrix metalloproteinases (MMPs) in skin fibroblasts, causing photo-aging and tumor progression. The MMP family is involved in the breakdown of extracellular matrix in normal physiological processes such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes such as arthritis and metastasis. We investigated the effect of diphlorethohydroxycarmalol (DPHC) against damage induced by UVB radiation in human skin keratinocytes. In UVB-irradiated cells, DPHC significantly reduced expression of MMP mRNA and protein, as well as activation of MMPs. Furthermore, DPHC reduced phosphorylation of ERK and JNK, which act upstream of c-Fos and c-Jun, respectively; consequently, DPHC inhibited the expression of c-Fos and c-Jun, which are key components of activator protein-1 (AP-1, up-regulator of MMPs). Additionally, DPHC abolished the DNA-binding activity of AP-1, and thereby prevented AP-1-mediated transcriptional activation. These data demonstrate that by inactivating ERK and JNK, DPHC inhibits induction of MMPs triggered by UVB radiation.

Key Words: Diphlorethohydroxycarmalol, Human skin keratinocytes, Matrix metalloproteinases, Photo-aging, Ultraviolet B

INTRODUCTION

The sun emits ultraviolet (UV) radiation in the UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm) wavelengths. The Earth's ozone layer blocks 97-99% of this UV radiation. Due to the ongoing depletion of the ozone layer, elevated levels of UV wave are penetrating the atmosphere and reaching the Earth's surface, leading to serious environmental problems. Overexposure to UVB radiation causes sunburn and some forms of skin cancer. However, the most deadly effect of UVB on cells is the result of indirect DNA damage mediated by free radicals and oxidative stress; consistent with this, 92% of all melanomas lack the signature mutations of direct UV damage (Davies *et al.*, 2002).

UV radiation can damage collagen fibers, thereby accelerating skin aging. In particular, UVB destroys vitamin A in the skin, which may cause further damage (Berne *et al.*, 1984).

In keratinocytes, UVB irradiation upregulates the expression of MMP-1, further contributing to premature skin aging (Kim *et al.*, 2010). MMP-1 also known as interstitial collagenase is one of the matrix metalloproteinases (MMPs) belongs to collagenases group, which plays important roles in physiological processes including embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling (Brinckerhoff and Matrisian, 2002). MMP-1, also known as interstitial collagenase, initiates cleavage of fibrillar collagen (type I and III in skin) at a single site within its central triple helix (Fisher *et al.*, 2002), contributing to the degradation of the extracellular matrix; thus, MMP-1 contributes to the etiology of many age-related degenerative diseases (Kähäri and Saarialho-Kere, 1997; Jacob, 2003). In many cancers, aberrant expression of MMP-1 is associated with both severe disease and poor patient outcome. Moreover, MMP-1 plays a prominent role in the proteolytic release and activation of growth factors, cytokines,

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*Corresponding Author

E-mail: jinwonh@jejunu.ac.kr
Tel: +82-64-754-3838, Fax: +82-64-702-2687

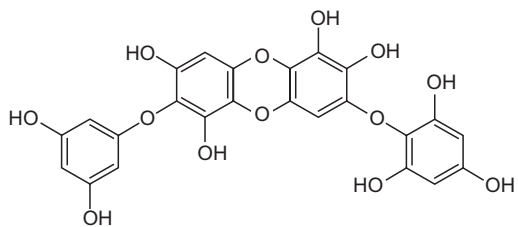


Fig. 1. The structure of Diphlorethohydroxycarmalol.

and signaling peptides that modulate the senescent micro-environment. Further, it has been reported that MMP-2 and MMP-9, belong to gelatinases group of MMPs are also can be expressed at elevated levels at oxidative stress (Reel *et al.*, 2009). The function of these gelatinases are to degrading type IV collagen, the most abundant component of the basement membrane which is important for maintaining tissue organization, providing structural support for cells, and influencing cell signaling and polarity. Basement membrane decomposition is a key step for the metastatic progression of most cancers (Mook *et al.*, 2004).

The regulatory mechanisms that control senescence-associated MMP-1 expression are unknown. Previously, we hypothesized that anti-oxidants play an important role in regulating MMP-1 expression (Kang *et al.*, 2008a,b). Redox activation of c-Jun-N-Terminal Kinase (JNK) is responsible for the age-dependent increases in MMP-1 expression, and this redox-sensitive signaling pathway controls activation of the AP-1 transcription factor and the chromatin remodeling events that lead to upregulation of MMP-1 (Dasgupta *et al.*, 2010). Oxidative stress also stimulates extracellular signal-regulated kinase (ERK), another important regulator of MMP expression. Inhibition of ERK abrogates Ras- and serum-induced stimulation of the MMP-1 promoter, indicating that ERK plays an essential role in the transcriptional regulation of MMP-1 (Frost *et al.*, 1994). Furthermore, overexpression of dominant-negative MEK blocks insulin-mediated induction of a reporter construct containing the activator protein (AP)-1 element from the MMP-1 promoter (Westermarck and Kähäri, 1999). These studies suggest that the ERK/AP-1 pathway is the major activator of MMP-1 expression. Moreover, ERK and JNK, which belong to the mitogen-activated protein kinase (MAPK) family, are associated with expression of MMP-1 in clinical material (Davidson *et al.*, 2003).

Antioxidants can effectively attenuate UV-induced cell damage and skin photoaging by suppressing cell apoptosis and expression of MMP-1 via MAPK signaling pathways (Ren *et al.*, 2010). Therefore, antioxidants might be useful as new anti-aging agents for photo-damaged skin (Jo *et al.*, 2012). Our previous studies showed that the antioxidant properties of diphlorethohydroxycarmalol (DPHC, Fig. 1) can reduce production of MMP-1 by inhibiting ERK and AP-1 (Kang *et al.*, 2008b). This compound protects cells against damage cause by UVB radiation, as demonstrated the fact that DPHC treatment decreases the length of the damaged DNA tail in a comet assay and prevents morphological changes in fibroblasts (Heo *et al.*, 2010). DPHC may inhibit α -glucosidase and α -amylase activities and alleviate postprandial hyperglycemia in streptozotocin-induced diabetic mice (Heo *et al.*, 2009). Moreover, DPHC exerts antioxidant effects against H_2O_2 -me-

diated damage, and treatment with this compound represents a potential therapeutic modality for several diseases associated with oxidative stress (Heo and Jeon, 2009). However, the protective effect of DPHC in terms of MMPs and UVB-associated senescence has not been thoroughly studied. Hence, in this study we focused on the ability of DPHC to protect against UVB-induced cellular senescence caused by MMPs activation, and the possible underlying mechanisms involved. A fundamental understanding of the mechanisms by which various antioxidant compounds block MMPs production is essential for the design of safe and effective therapeutic strategies for the treatment of many age-associated degenerative diseases.

MATERIALS AND METHODS

Reagents

DPHC was dissolved in dimethyl sulfoxide (DMSO) and final concentration in control or DPHC treatment did not exceed the 0.05%. Primary MMP-1 antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA). Primary antibodies against MMP-2 and -9 were purchased from Abcam (Cambridge, MA, USA). Primary antibodies against ERK 2, phospho ERK 1/2, mitogen-activated protein kinase kinase (MEK) 1, phospho MEK 1, JNK 1/2, phospho JNK 1/2, stress-activated protein kinase/extracellular signal-regulated kinase (SEK) 1, phospho SEK 1, c-Fos, c-Jun and phospho c-Jun were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against actin was from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Cell culture

Human HaCaT keratinocytes (Amore Pacific, Gyeonggi-do, Republic of Korea) were maintained at 37°C in an incubator with a humidified atmosphere of 5% $CO_2/95\%$ air. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B.

Drug treatment and UVB irradiation

HaCaT cells were seeded at 1×10^5 cells/ml, allowed to adhere to the culture plate, and then treated with DPHC. After 1 h, the cells were exposed to ultraviolet B (UVB) at 150 mJ/cm². The UVB source was a CL-1000M UV Crosslinker (UVP, Upland, CA, USA), which delivered UVB energy at wavelengths ranging from 280 to 320 nm (peak intensity, 302 nm).

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from cells using easy-BLUE (Intron Biotechnology, Daegjeon, Republic of Korea). The polymerase chain reaction (PCR) conditions for MMP-1 and the house-keeping gene GAPDH were as follows: 35 cycles of 94°C for 45 sec, 52°C for 1 min, and 72°C for 1 min. Primer pairs (Bionics, Seoul, Republic of Korea) were as follows (forward and reverse, respectively): MMP-1, 5'-GGAGGAAATCTTGC-TCAT-3' and 5'-CTCAGAAAGAGCAGCATC-3'; GAPDH, 5'-AAGGTCGGAGTCAACGGATTT-3' and 5'-GCAGTGAGGGTCTC-TCTCCT-3'. The amplified products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV illumination.

Immunoblot analysis

The cells were harvested, washed twice with PBS, lysed on ice for 10 min in 100 μ l PRO-PREP™ protein extraction solution (Intron Biotechnology), and centrifuged at 13,000 rpm for 5 min. The resultant supernatants were collected, and the protein concentrations were determined. Aliquots of the lysates (20 μ g of protein) were boiled for 5 min and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose membranes, which were then incubated with primary antibodies. Next, the membranes were incubated with secondary immunoglobulin G-horse radish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and then exposed to X-ray film.

Determination of MMP-1 activity

Secreted active MMP-1 was measured using the Human Active MMP-1 Fluorokine® E Kit (R&D Systems, Minneapolis, MN, USA). HaCaT cells were treated with 20 μ M of DPHC for 1 h, after the 150 mJ/cm² of UVB exposure and the samples were incubated for 1 day. Culture medium was subjected to centrifugation at 1,000 \times g for 5 min, and then 150 μ l of culture supernatants was mixed with 100 μ l of assay diluent buffer in 96-well enzyme-linked immunosorbent assay (ELISA) plates. The plates were shaken for 3 h at room temperature, and then unbound material was washed off. Subsequently, 200 μ l of activation reagent (0.5 M APMA in DMSO) was added to each well for pro-MMP-1 activation. The plates were incubated for 2 h at 37°C in a humidified environment. After washing, 200 μ l of fluorogenic substrate was added. After another 20 h at 37°C, fluorescence was measured using FLUOstar Optima Microplate Reader (BMG Labtech, Cary, NC, USA) with the excitation wavelength set to 320 nm and the emission wavelength set to 405 nm (Tsareva *et al.*, 2007).

Determination of MMP-2 and MMP-9 activities

MMP-2 and MMP-9 enzymatic activities in serum free culture medium were determined by SDS-PAGE gelatin zymography. Gelatin zymography was performed using the Novex In-gel Zymography system (Invitrogen), according to the manufacturer's instructions. Protein concentrations were analyzed with a Bio-Rad (Hercules, CA, USA) system. Equal amounts of medium for each sample were mixed with 2 \times sample buffer and loaded on a Novex 10% Zymogram Gelatin Gel for electrophoresis. After electrophoresis, gels were followed by incubation with Zymogram Renaturing Buffer, and then with Zymogram Developing Buffer. After an overnight reaction, the gel was stained with SimplyBlue Safestain (Invitrogen). After staining, the gels were destained appropriately. Gelatinolytic activity was visualized as a transparent band against a blue background. Gelatinolytic bands were measured densitometrically with an image analyzer (Epson GT-9500 scanner).

Chromatin immunoprecipitation (ChIP) analysis

ChIP assays were performed using the SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA), with slight modifications to the manufacturer's protocol. Cells were cross-linked by the addition of 1% formaldehyde. Chromatin was prepared and digested with nuclease for 12 min at 37°C. c-Jun antibody, normal rabbit IgG and histone

H3 (D2B12) XP™ antibody were added to the chromatin digests, which were incubated with constant rotation overnight at 4°C. ChIP-grade protein G magnetic beads were added to capture the immunoprecipitated complexes. The beads were washed, and the immunoprecipitates were eluted with ChIP elution buffer. Cross-links were reversed by incubation of the eluent at 65°C for 30 min, followed by addition of proteinase K and incubation at 65°C for an additional 2 h. The immunoprecipitated DNA fragments were then purified on spin columns. DNA recovered from the immunoprecipitated complex was subjected to 35 cycles of PCR. The primers for the *MMP-1* gene promoter were as follows: forward, 5'-CCT CTT GCT GCT CCA ATA TC-3'; reverse, 5'-TCT GCT AGG AGT CAC CAT TTC-3' (Goffin *et al.*, 2010). The PCR products were separated on 2% agarose gels, and DNA bands were visualized using ethidium bromide staining and Image Quant 350 (GE Healthcare, Wankesha, WI, USA).

Transient transfection and AP-1 luciferase assay

Cells were transiently transfected with a plasmid harboring the AP-1 promoter and the *Renilla* promoter using Lipofectamine™ 2000 (Invitrogen, Eugene, OR, USA). Following overnight transfection, the cells were treated with 20 μ M of DPHC, incubated for 1 h, and then exposed to UVB at 150 mJ/cm². After 24 h, the cells were washed twice with PBS and lysed with passive lysis buffer (Promega, Madison, WI, USA). Following vortex mixing and centrifugation at 12,000 \times g for 30 sec at 4°C, the supernatant was stored at -70°C. For the luciferase assay, 20 μ l of cell extract was mixed with 100 μ l of the luciferase assay substrate reagent at room temperature, and the mixture was placed in an illuminometer to measure the light produced. Next, 100 μ l of *Renilla* substrate reagent was added at room temperature, and the light produced was again measured using the illuminometer. AP-1 luciferase activities were calculated by dividing luciferase light production by *Renilla* light production.

Statistical analysis

All measurements were performed in triplicate, and all values are represented as means \pm standard error. The results were subjected to an analysis of variance (ANOVA) using the Tukey test; $p < 0.05$ was considered to represent a significant difference.

RESULTS

DPHC down-regulates UVB-induced MMP-1 expression and activation

UVB irradiation of skin fibroblasts induces generation of reactive oxygen species (ROS), which promote transcription and expression of MMP-1, causing skin photoaging and skin tumor progression (Brenneisen *et al.*, 1997). UVB irradiation markedly increased the MMP-1 mRNA levels, as demonstrated by RT-PCR analysis; DPHC pretreatment decreased the level of MMP-1 transcript induced by 150 mJ/cm² of UVB irradiation (Fig. 2A). Consistent with the RT-PCR results, immunoblots confirmed that DPHC decreased the induction of MMP-1 protein expression by UVB irradiation (Fig. 2B). Moreover, DPHC significantly decreased the amount of active MMP-1 (Fig. 2C). Further western blot analysis revealed that DPHC treatment diminished the expression of both MMP-2 and MMP-9 (Fig.

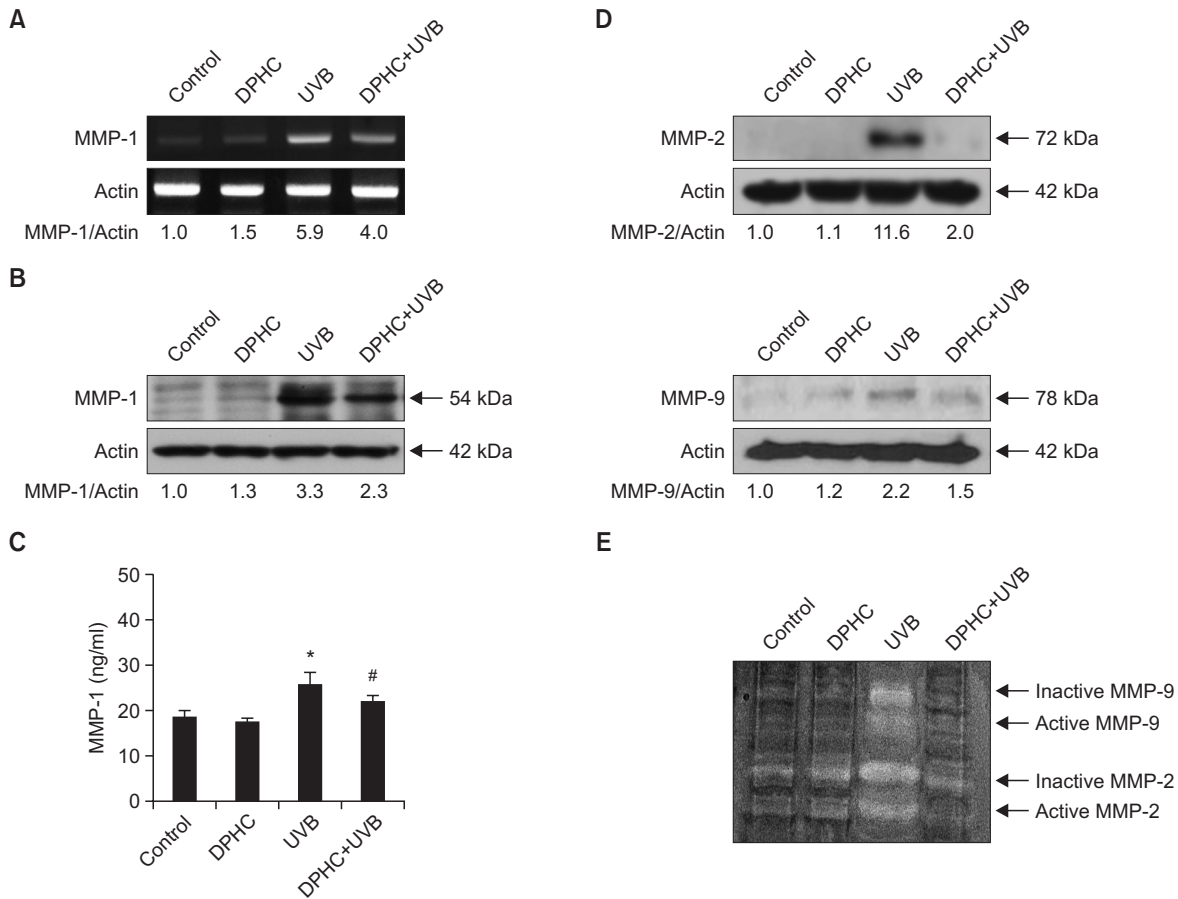


Fig. 2. The effects of DPHC on UVB-induced MMPs transcription, expression, and activation. (A) MMP-1 mRNA levels were measured by reverse-transcription (RT) PCR analysis. Cells were treated with 20 μ M of DPHC for 1 h, and then irradiated with 150 mJ/cm² UVB. After UVB irradiation, cells were incubated for 24 h in a humidified incubator containing 5% CO₂ at 37°C, and the cells were then harvested for RT-PCR. (B, D) Cell lysates were electrophoresed, and MMP-1, -2 and -9 protein expression of was detected by immunoblotting. (C) Active MMP-1 was quantitated in culture media supernatants. Cells were incubated in serum-free media to eliminate interference from MMP-1 in serum. *Significantly different from control cells ($p < 0.05$); #significantly different from UVB-irradiated cells ($p < 0.05$). (E) Inactive and active MMP-2 and -9 were assessed by using gelatin zymography. Top two bands indicate MMP-9 gelatinase and lower two bands indicate MMP-2 gelatinase.

2D). Using gelatin zymography, activation of MMP-2 and MMP-9 enzymes were measured in UVB irradiation-exposed cells. Zymography analysis revealed the presence of inactive and active forms of MMP-2 and MMP-9 in UVB irradiation-exposed cells (Fig. 2E). However, DPHC pre-treated cells showed reduction of inactive and active forms of MMP-2 and MMP-9 (Fig. 2E). These data indicate that DPHC significantly decreased activation of MMP enzymes.

DPHC reduces the ERK and JNK activation in response to UVB irradiation

The AP-1 site plays a critical role in the transcriptional activation of MMP promoters (Crawford and Matrisian, 1996; Benbow and Brinckerhoff, 1997). AP-1, a heterodimeric protein consisting of proteins belonging to the Fos and Jun families, is a transcription factor, capable of binding to 5'-TGAGTCA-3' DNA elements (Farrell *et al.*, 1989; Glover and Harrison, 1995). The Fos and Jun families are regulated by MAPKs, especially ERK and JNK (Cano *et al.*, 1994; Murphy *et al.*, 2002; Bogoyevitch *et al.*, 2010). As shown in Fig. 3A and B, DPHC

markedly suppressed the activation of ERK and JNK by UVB irradiation. DPHC also reduced MEK1 and SEK1 expressions. Consequently, UVB-induced activation of c-Fos and phospho-c-Jun was attenuated by DPHC (Fig. 3C). These results suggested that UVB induction of AP-1 components, which are controlled by ERK and JNK activation, are attenuated by DPHC treatment.

Suppression of AP-1 transcriptional activity by DPHC treatment

Next, we performed a ChIP assay to investigate the effect of DPHC on AP-1 transcriptional activity. The results revealed that AP-1 transcriptional activity was higher in UVB-irradiated cells than in controls (Fig. 4A). However, DPHC pretreatment significantly attenuated AP-1 transcriptional activity in UVB-exposed HaCaT cells. In addition, we assessed AP-1 transcriptional activity using the luciferase reporter assay. Consistent with the ChIP assay data, the reporter assay showed that UVB-induced elevation of AP-1 transcriptional activity was significantly downregulated by DPHC pretreatment. These in-

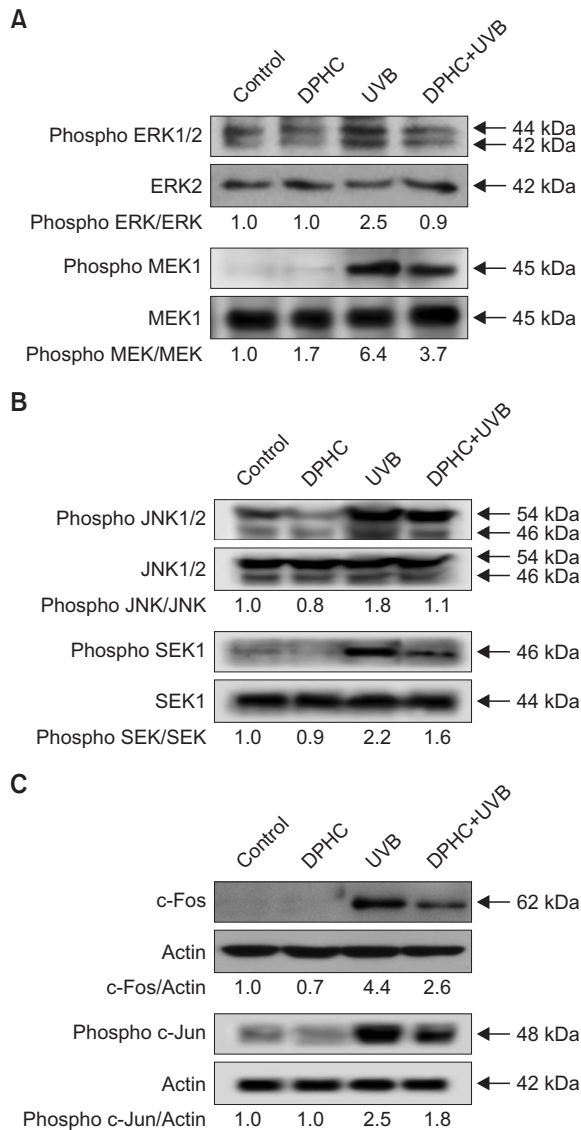


Fig. 3. Effects of DPHC on c-Fos and c-Jun expression via activation of ERK and JNK signal transduction. (A) Cell lysates were electrophoresed and immunoblotted using anti-phospho ERK1/2, anti-ERK2, anti-phospho MEK1, and MEK1 antibodies. (B) Cell lysates were electrophoresed and immunoblotted using anti-phospho JNK1/2, anti-JNK1/2, anti-phospho SEK1, and SEK1 antibodies. (C) Cell lysates were electrophoresed and immunoblotted using c-Fos and phospho c-Jun antibody, respectively.

vestigations suggested that DPHC reduced UVB induction of MMP-1 by suppressing AP-1 transcriptional activity (Fig. 4B).

DISCUSSION

Proteins of the MMP family are involved in the breakdown of extracellular matrix (ECM) during multiple normal physiological processes such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes such as arthritis and metastasis. In cancer metabolism, MMP-1 expression may be useful as a novel marker for hematogenous

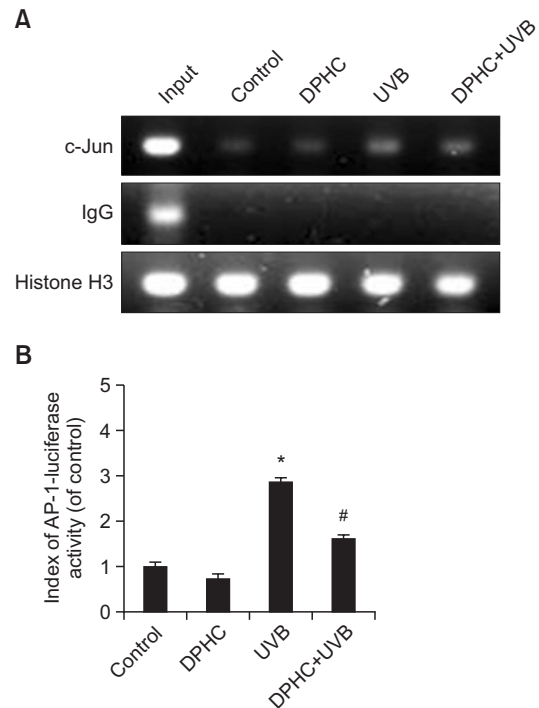


Fig. 4. Effects of DPHC on AP-1 transcriptional activity. (A) Cellular extracts were prepared from HaCaT cells treated with 20 μ M of DPHC for 1 h, and then irradiated with 150 mJ/cm^2 UVB. After UVB irradiation, cells were incubated for 24 h, ChIP assay was performed to assess AP-1 binding to the MMP-1 promoter (B) Cells were transfected with plasmid containing the AP-1 binding site/luciferase construct. After transfection, cells were treated 20 μ M of DPHC for 1 h, irradiated with 150 mJ/cm^2 UVB dose, and incubated for 24 h. *Significantly different from control cells ($p < 0.05$); #significantly different from UVB-irradiated cells ($p < 0.05$).

metastasis of colorectal cancer, and its inhibition may be a strategy for prevention of metastasis (Sunami *et al.*, 2000). As the range of proteolytic targets of MMPs are more complex. MMP inhibitors (MMPi) are potentially useful for blocking cancer progression, but at the same time could suppress normal tissue function or host defense processes. Therefore in the process of developing MMPi it is necessary to focus on exact MMPs and their transcription, secretion of the zymogen into the extracellular matrix, and activation of the zymogen (Purcell *et al.*, 2002). Moreover, MMP-1 causes UVB-mediated senescence and cancer (Westermarck and Kähäri, 1999; Sunami *et al.*, 2000; Wenham *et al.*, 2003; Yaar and Gilchrist, 2007). UVB-irradiated keratinocytes, which express elevated levels of IL-1 α and IL-6, stimulate MMP-1 production by human fibroblasts, suggesting that UVB irradiation modulates MMP-1 production by both direct and indirect mechanisms (Fagot *et al.*, 2002). Additionally, many reports have suggested that ROS causes senescence in the skin via activation of MMP-1 (Wenk *et al.*, 1999; Brenneisen *et al.*, 2002; Kang *et al.*, 2008b). DPHC can alleviate oxidative stress both directly and indirectly (Heo *et al.*, 2010; Heo *et al.*, 2009; Heo and Jeon, 2009). In this study, we showed that DPHC could suppress MMP-1 expression resulting from UVB-induced cell damage and ROS generation (Fig. 2). Moreover, DPHC down-regulated UVB-induced MMP-1 expression and activation in human keratinocytes (Fig. 2). Similarly MMP-2 and MMP-9 are also

important in cancer, which are proven to be expressed at higher concentrations at oxidative stress. Western blot and zymography analysis confirmed that UVB-induced over expression of gelatinases were abrogated by DPHC (Fig. 2).

Overall, oxidative conditions promote expression of MMP-1 via activation of the AP-1 transcription factor (Crawford and Matrisian, 1996; Wenk *et al.*, 1999; Chen *et al.*, 2004). AP-1 complexes bind to the promoter regions of the MMP genes and regulate MMP gene expression (Lin *et al.*, 1993). c-Fos and c-Jun, the key components of AP-1, are primarily transcriptionally regulated by phosphorylated MEK/ERK and SEK/JNK, respectively (Kieser *et al.*, 1997; Eliopoulos and Young, 1998; Favata *et al.*, 1998; Wu *et al.*, 2002). Further it has been proven that JNK inhibition prevents UV-induced c-Jun phosphorylation while ERK inhibition prevents UV-induced c-Fos expression in human dermal fibroblasts (Kim *et al.*, 2005). Aligning with these findings our data revealed that DPHC suppressed UVB-induced phosphorylation of MEK/ERK and SEK/JNK (Fig. 3) and reduced UVB-induced AP-1 transcriptional activity in the MMP-1 promoter region (Fig. 4); together, these observations suggest a mechanism by which MMP-1 expression and activation are down-regulated by DPHC treatment.

In summary, the results of this study suggest that DPHC protects HaCaT cells against senescence-associated MMP-1 as well as MMP-2 and MMP-9 induced by UVB irradiation. In HaCaT cells treated with DPHC, activation of ERK and JNK by UVB radiation was dramatically decreased, resulting in lower DNA-binding activity of AP-1 and ultimately to reduced MMPs expression in response to UVB.

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