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Effects of Neo-endorphin on Anti-aging and Autophagy Activation in Human Dermal Fibroblast Cells

조선대학교 대학원

생명과학과

임 가 은

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인간 피부 섬유아세포에서 항노화 및 **autophagy** 활성화에 미치는 네오엔돌핀의 영향에 관한 연구

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조선대학교 대학원

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CONTENTS

LIST OF FIGURES

ABSTRACT

Effects of Neo-endorphin on Anti-aging and Autophagy Activation in Human Dermal Fibroblast Cells

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Skin aging is influenced by several genetic, physiological and environmental factors, among which solar ultraviolet (UV) irradiation is one of the most important causes for skin photoaging. Autophagy, a process that maintains homeostatic balance between the synthesis, degradation, and recycling of cellular organelles and proteins, plays an important regulatory role in differentiation, development, stress, diseases, cancer, and aging. Recent studies have reported that the increased autophagy can delay aging and also extend longevity. One of opioid neuropeptides, α-neoendorphin (NEP) is an endogeneous decapeptide (N-YGGFLRKYPK-C) and activates kappa opioid receptor for its signaling. Although there are some reports on NEP's anti-aging and anti-wrinkling effects on skin cells, its action mechanism is not well-known yet in detail. In this study, the effects of NEP on anti-skin aging and autophagy activation in human dermal fibroblast cells (called Hs68) irradiated with UVB (35 mJ/㎠) were investigated. When Hs68 cells were treated with NEP (5 ㎍/㎖) for 15 min, MAP kinase signaling pathway could be activated, as judged by

Western blottings using antibodies raised against p-ERK, ERK, p-p38, p38, p-JNK, and JNK. In addition, the expression levels of autophagy-related proteins, including Beclin-1, Atg12 and LC3 I-II were increased in Hs68 cells treated with NEP (5 ㎍/㎖) for 30 min, accompanied with the decreased level of mTOR. The ability of NEP to activate the autophagy system also could be confirmed by the confocal microscopy using an immunostaining with anti-LC3 antibody that NEP (5 ㎍/㎖) could induce the formation of autophagosomes in Hs68 cells irradiated with UVB (35 mJ/㎠). These results suggest that NEP can activate the autophagy system through the activation of MAP kinase signaling pathway in cells irradiated with UVB. In addition, NEP could decrease the expressions of skin aging-related proteins, such as ERK, p38, and JNK, suggesting that it can exert anti-aging effects against photo-aging in skin. NEP (5 ㎍/㎖) also could inhibit the production of reactive oxygen species (ROS) in the Hs68 cells irradiated with UVB (35 mJ/㎠), as judged by measuring the intensity of the fluorescence emitted from 2′,7′-dichlorofluorescin diacetate (DCFH-DA). These results suggest that NEP can show the anti-aging effects on UV-irradiated skin through the decreases of the expression/production levels of aging-related proteins and ROS. On the other hands,

The results of RT-PCRs showed that the transcription level of type I procollagen gene could be increased to approximately 10%, whereas that of matrix metalloproteinase (MMP)-1 gene could be decreased to about 46% by the treatments with NEP (5 ㎍/㎖) for 24 h in Hs68 cells irradiated with UVB (35 mJ/㎠) in a dose-dependent manner. In fact, the relative synthesis of collagen increased to an average of 32.4% by the NEP treatment through its inhibitory effects on collagenase activity in UVB-irradiated Hs68 cells. All these results suggest that NEP may reduce the formation of ROS by activating the autophagy system and also prevent from UVB-induced extracellular matrix degradation by inhibiting the expression of MMP-1 through the inhibition of MAP kinase signaling pathway. Taken together, all the results obtained by this study demonstrate that NEP can be a potential anti-skin photoaging agent.

Ⅰ. INTRODUCTION

Human skin that is composed of epidermis, dermis and hypodermis plays a variety of physiological function like regulating of body temperature and defending against the external environment condition (Rittie *et al.*, 2002). The epidermis is mainly composed of melanocytes, langerhans cells, and merkel cells (Langton *et al.*, 2010). The major components of the dermis are fibroblasts which produce collagen and elastic fibers. Collagen and elastin support the skin structure by forming crosslinks (Bouwstra *et al.*, 2002).

Skin aging is divided into intrinsic and extrinsic aging processes (Fisher *et al.*, 2002) (Fig. 1). The intrinsic aging is naturally occurred by genetic factors as we grow older and the extrinsic aging is caused by external factors such as smoking, wind, and heater. In particular, an exposure to ultraviolet (UV) light is the most influential factor in skin aging (Bryant *et al.*, 2000). UV irradiation causes sunburn, photoaging, immune suppression and more seriously cancer on human skin (Park *et al*., 2010). Especially, UVB (wave-length of 320 ~ 290 nm) generate reactive oxygen species (ROS) in skin cell, which leads to activate mitogen-activated protein kinases (MAPKs), including ERK, p38, and JNK (Park *et al*., 2012). The activated MAP kinases increase the activities of the matrix metalloproteinases (MMPs) (Hwang *et al*., 2011). Among the MMPs, matrix metalloproteinases-1 (MMP-1) plays a major role in the degradation of collagen, since this is a major cause for the dermal matrix breakdown that occurs in photoaging (Chiang *et al*., 2012) (Fig. 2).

Autophagy is a process that maintains the protein homeostasis and eliminates cellular waste components such as proteins, lipids, sugars, nucleic acids and organelles (Levine *et al*., 2008). The system is generally activated under a nutrient deprivation, but also its activation is associated with physiological as well as pathological precesses, including development, differentiation, neurodegenerative

diseases, stress, infection, cancer and aging (Lemasters *et al*., 2005) (Fig. 3). The regulation of the autophagy system is very complex process, in which mTOR is a critical regulator (Faghiri *et al*., 2010). The activated mTOR suppresses autophagy activation, whereas the deactivated mTOR promotes it (Alers *et a*l., 2012). Beclin-1 interacts with Bcl-2, inhibiting the autophagy induction. Beclin-1 released by the phosphorylated Bcl-2 through the activated MAPKs (ERK, p38, JNK) induces the autophagy activation (Zhang *et al*., 2008) and Atg proteins also participate in regulating the system (Levine *et al.,* 2008). Atg12 is combined to Atg5, then interacts with Atg16 to form a large complex. In addition, microtubule-associated protein 1 light chain 3 (MAP-LC3) is a central protein in the autophagy system. The conversion of LC3-Ⅰ to LC3-Ⅱ is an indicator for the formation of autophagosome in the induction of autophagy (Codogno *et al*., 2012). In practice, LC3-Ⅱ is used as an autophagosomal marker protein, because the molecule is located in the surface of the membrane of autophagosome (Levine *et al.,* 2008) (Fig. 4).

Generally, the autophagic ability declines with age (Cuervo *et al*., 2008), as indicated by the impairment of autophagy for organelles and autophagosomes (Terman *et al*., 1995).

Many studies show that autophagy inductors, including Atg proteins and Beclin-1, trigger autophagic degradation with age (Rubinsztein *et al*., 2011). The accumulation of abnormal proteins lead to decline cellular functions and eventually cause age-related diseases, including the Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and cancer (Selkoe *et al*., 2011, Finkbeiner *et al*., 2011, Taylor *et al*., 2011, Schmidt *et al*., 2014). The autophagic activity is also more reduced in dermal firbroblasts derived from elderly women than in the same cells from young women (Fig. 5). Moreover, the increased collagen, elastin, and hyaluronic acid can be observed when the autophagic degradation is suppressed in aged skin tissue (Kanae *et al*., 2013).

Skin cells, including keratinocytes, melanocytes, langerhans cells, and fibroblasts, are influenced by neurotransmitters (Roosterman *et al.*, 2006). The neurotransmitters are the molecules that allow the transmission of signals across synapses between neurons (George *et al.*, 2009).

Neuropeptides included in neurotransmitters are enkephalin, α-neoendorphin, β-neoendorphin, α-endorphin, β-endorphin, ɣ-endorphin, and dynorphin *etc* and they bind to specific receptors (Hollt *et al.*, *1986*). Among them, α-neoendorphin that is an endogenous opioid peptide is a decapeptide consisted of H_2N-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-COOH having a molecular weight of 1228.4 Da (Kangawa *et al.*, 1981) (Fig. 6). Neoendorphins can directly bind to the kappa opioid receptor (KOR) on the plasma membrane of skin cells, such keratinocytes and fibroblasts (Werz *et al.*, 1985) to induce a variety of biological events, including wound healing and skin retexturizing (Brem *et al.*, 2007).

Therfore, this study was performed to reveal the followings: 1) the autophagy activation ability of NEP in human dermal fibroblast cells exposed to UVB light; 2) the effects of NEP on skin photo-aging through by modulating MAP kinase signaling pathway.

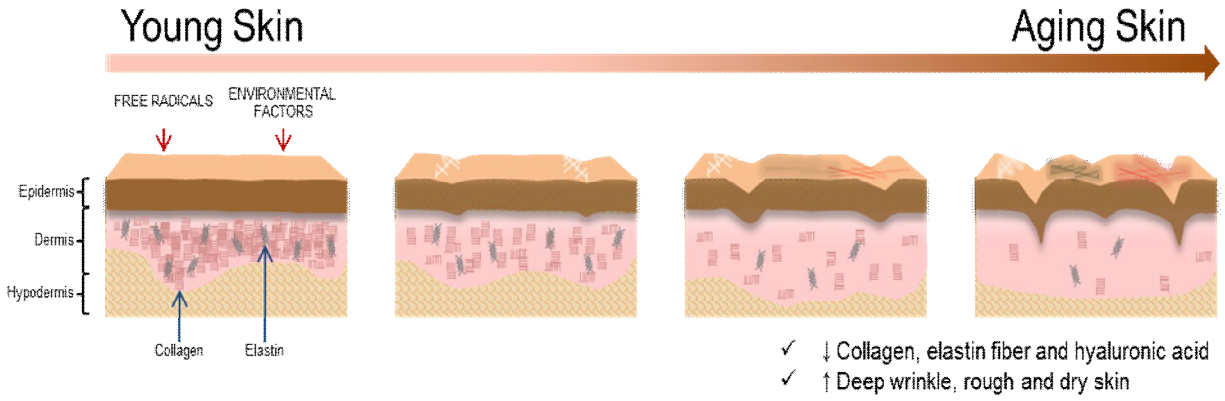

Fig. 1. Schematic diagram showing the structure of skin and the process of skin aging. Human skin consists of epidermis, dermis, and hypodermis. The major components of the dermis are fibroblasts which produce collagen (~90%) and elastic fibers. Collagen, elastin fiber, and hyaluronic acid decrease, resulting in deep wrinkle, rough and dry skin in process of skin aging. Modified from Langton *et al.*, (2010).

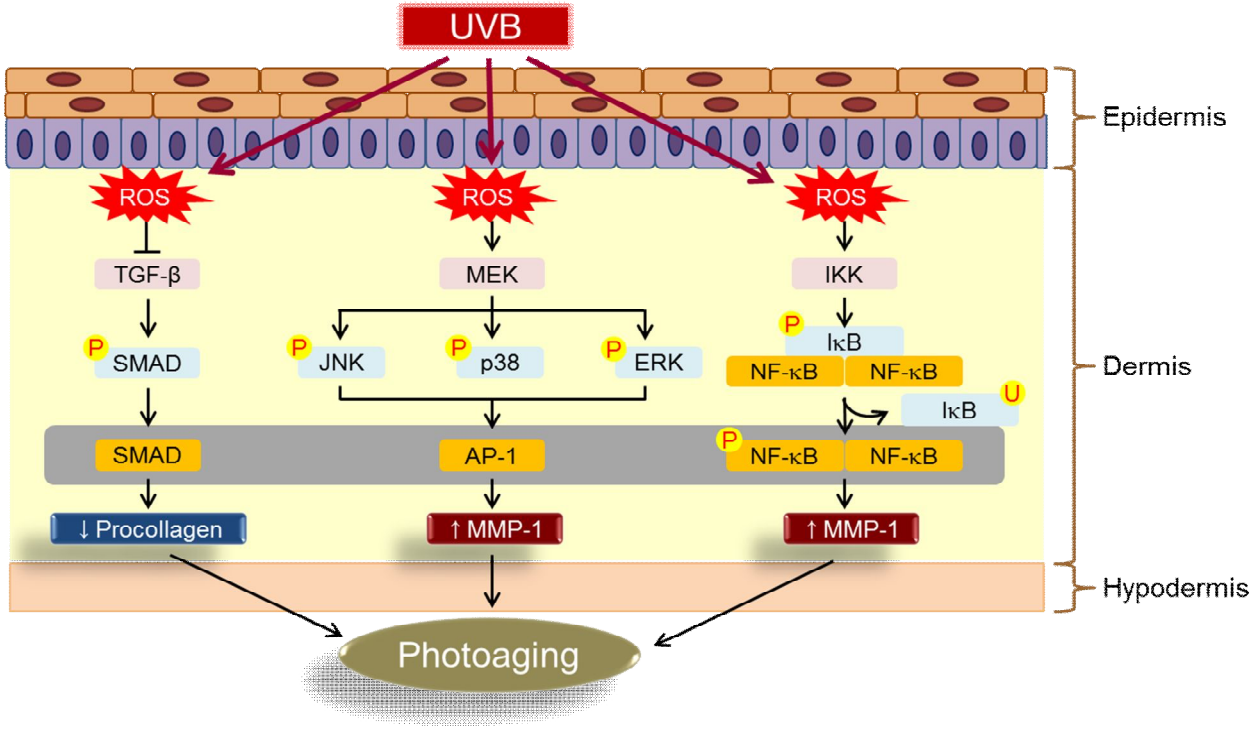

Fig. 2. Photoaging-inducing mechanism in skin. UVB irradiation generates ROS in skin cell, which leads to activate MAP kinase. The activated MAP kinases increase the productions of MMPs. Among them, MMP-1 plays major role in the degradation of collagen, which is the major cause of the dermal matrix breakdown occurring in photoaging.

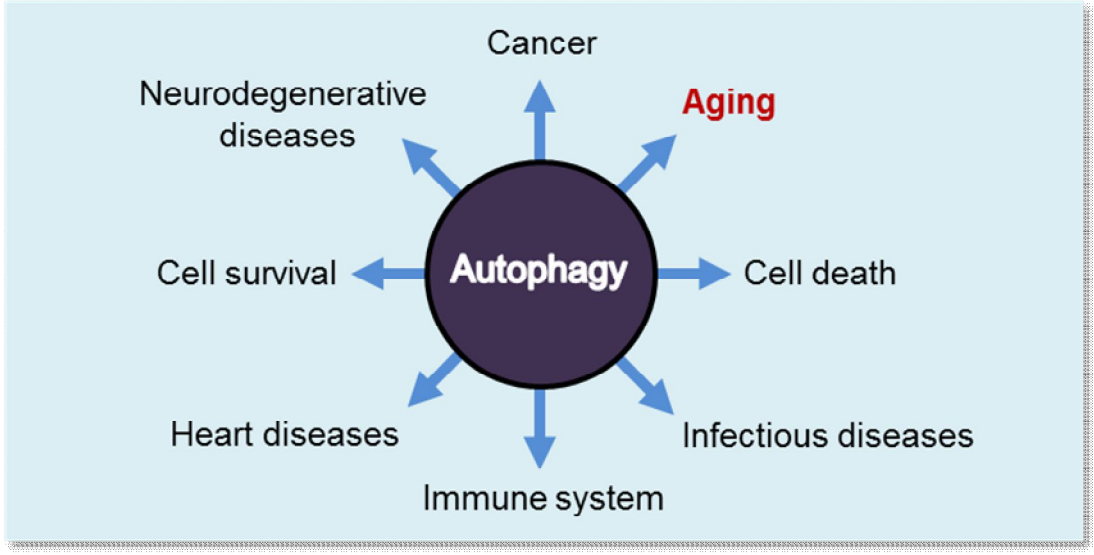

Fig. 3. Schematic diagram of autophagy system. Autophagy, a process that maintains homeostatic balance between the synthesis, degradation, and recycling of cellular organelles and proteins, plays an important regulatory role in differentiation, development, stress, diseases, cancer, and aging. Modified from Lemasters *et al.*, (2005).

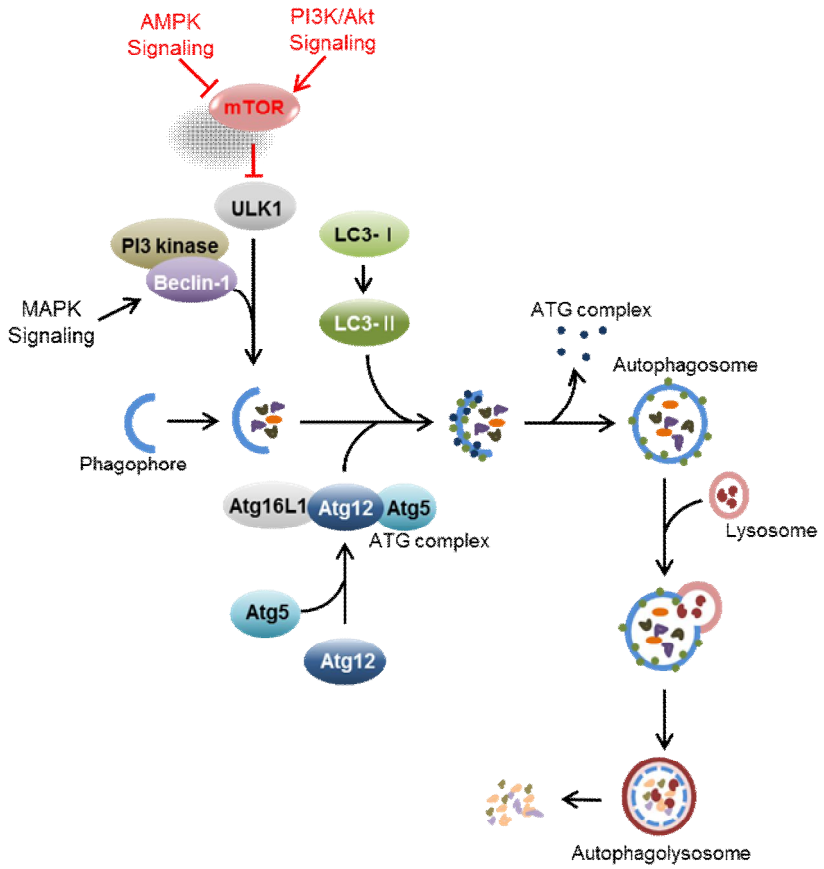

Fig. 4. Autophagy signaling pathway. The regulation of autophagy is very complex and the mTOR is a critical regulator for the autophagy induction. The decreased mTOR activates ULK1 to recruit Beclin-1 that is activated MAP kinases to initiate the autophgy. Atg12 is combined to Atg5, then interacts with Atg16 to form a large complex. Beclin-1 and Atg complex combines membrane. The conversion of LC3-Ⅰ to LC3-Ⅱ is indicative process for the formation of autophagosome to be fused to lysosome. Modified from Faghiri *et al*., (2010), Zhang *et al*., (2008), Levine *et al.,* (2008), and Levine *et al.,* (2008).

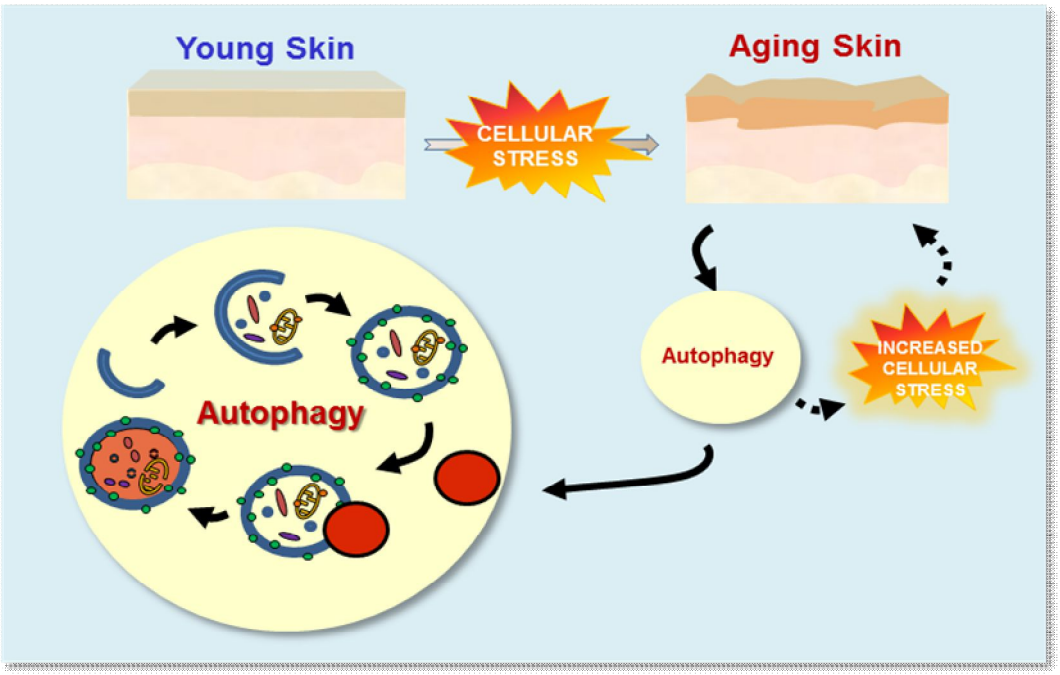

Fig. 5. The autophagy system of age-related skin. The autophagic activity is more reduced in dermal firbroblasts derived from elderly women than in the same cells from young women. Moreover, the increased collagen, elastin, and hyaluronic acid are occurred by suppressing the autophagic degradation in aged-skin tissue. Modified from Kanae *et al.*, (2013).

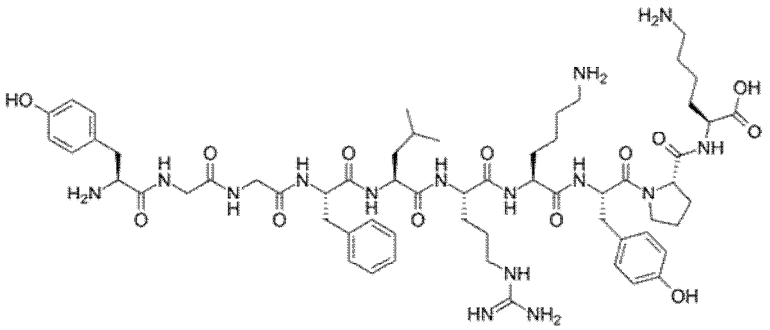

Fig. 6. Chemical structure of Neo-endorphin (NEP). NEP, an endogenous opioid peptide is composed of ten amino acids (H_2N-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-COOH) and its molecular weight is 1228.4 Da. (Kangawa *et al.*, 1981)

Ⅱ. MATERIALS AND METHODS

Ⅱ-1. Materials

Neo-endorphin (NEP) was obtained from Bio FD&C (Incheon, Korea). Dulbecco's modified Eagle's medium (DMEM; Lonza, Swiss), fetal bovine serum (FBS; ATLAS, USA), penicillin-streptomycin (Sigma, USA) were routinely used for cell culture. Antibodies against phospho-p38, p38, phospho-ERK, ERK, phospho-JNK, JNK, phospho-mTOR, mTOR, phospho-p70S6K, p70S6K, Beclin-1, and Atg12 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against LC3, KOR, GAPDH, goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (CA, USA). Alexa 488 was purchased from Invitrogen (USA). Collagenase (from *Costridium histolyticum*), 4-phenylazo-benzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg trifluoroacetate salt, 2′,7′-dichlorofluorescin diacetate, poly-L-lysine, and other reagents were purchased from Sigma (USA). TGF-β1 was from R&D systems (USA).

Ⅱ-2. Cell culture

Human skin fibroblast (Hs68) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 100 U/㎖ penicillin, and 100 ug/㎖ streptomycin in a 5% CO_2 incubator at 37℃. The cells were maintained until 60% to 80% confluency and used until the passages of 12 ~ 18.

Ⅱ-3. UVB irradiation

The Hs68 cells were irradiated with a UVB lamp (UVB-18, Claremont, CA) with a wavelength range of 280 ~ 315 nm, in which the energy density (35 mJ/cm^2) was adjusted using a UV radiometer (VLX-3.W; Vilber Lourmat, France).

Ⅱ-4. Cell viability assay

Cell viability was determined using non-radioactive cell proliferation assay kit (Promega, USA) according the manufacturer's instruction. Briefly, Hs68 cells cultured in 96-well plates (1×10^5 cells/well) were treated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and exposed to UVB (35 mJ/㎠), and then incubated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h in serum-free medium. After the treatments, 15 ㎕ of MTT dye solution was added to each well, and the cells were then cultured for 4 h at 37℃. Next, 100 ㎕ of solubilization/stop solution was added to each well, and then the cells were cultured for 1 h at 37℃. Absorbance of formazan products was measured at 570 nm using a microplate spectrophotometer (Molecular Devices Crop., Orleans, CA, USA).

Ⅱ-5. Western blot analysis

To investigate autophagy activation, Hs68 cells cultured in 6-well plates (0.6x10^5 cells/well) were treated with NEP (5 ㎍/㎖) or TGF-β (10 ng/㎖) for 15 min or 30 min at 37℃ in serum-free medium. To examine effects of anti-aging, the cells cultured in 6-well plates (0.6×10^5 cells/well) were pretreated with NEP (5 ㎍/㎖) or NAC (2 mM) for 24 h in serum-free medium. After exposed to UVB (35 mJ/㎠), the

cells were post-treated with NEP (5 ㎍/㎖) or NAC (2 mM) for 24 h. After the treatments, the cells were washed with PBS and lysed with ProNA CETi lysis buffer (TransLab, Korea). The protein concentrations were measured by Bradford method as described elsewhere. Routinely, 15 ㎍ of proteins were separated on 8%, 12%, or 15% SDS-polyacrylamide gels and transferred onto a PVDF membrane (Bio-Rad, CA, USA). The membrane was washed in TBS-T buffer (25 mM Tris-HCl, pH 8.0, 0.1% tween 20) and blocked in blocking reagent (5% skim milk in TBS-T) for 2 h at room temperature on a shaker. The membrane was incubated with primary antibodies (1:500 in the blocking buffer) overnight at 4℃. The membrane was washed with TBS-T for six times, and incubated with secondary antibodies (1:4000 in the blocking buffer) for 2 h at RT. 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 co., Ltd., Seoul, Korea) and exposed on X-ray film (Fuji Film, Japan). Western blot data was quantified using the program ImgeJ (National Institutes of Health, Bethesda, Maryland, USA).

Ⅱ-6. Confocal microscopic analysis

Hs68 cells irradiated or non-irradiated with UVB (35 mJ/㎠) were cultured (0.4×10^5 cells/well) on poly-L-lysine (0.1% solution)-coated glass coverslips in 6-well plates and treated with NEP (5 ㎍/㎖) or TGF-β (10 ng/㎖) for 30 min at 37℃. The cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 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 3% BSA in PBS for 20 min at RT and incubated with a 1:50 dilution of anti-LC3 antibody for overnight at 4℃. After washing three

times with PBS, cells were incubated with fluorescein (Alexa Fluor 488; Invitrogen, USA)-tagged antibody (diluted to 1:200 in PBS) 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).

Ⅱ-7. Measurement of the production of ROS

A fluorometric assay based on the oxidation of non-fluorescent DCFH-DA to highly fluorescent 2',7'-dichlorofluorescin (DCF) in the presence of esterases and ROS. To measure the generation of intracellular ROS in human skin fibroblasts, the cells were incubated at a density of 0.2×10^5 cells/well in a 48-well plates and treated with NEP (5 ㎍/㎖) or NAC (2 mM) for 0.5 h or 48 h in serum-free medium. The cells were washed once with PBS and exposed to UVB (35 mJ/㎠). The cells were treated with DCF-DA (20 μM) in serum-free medium and incubated for 30 min at 37℃ in 5% CO_2. After washing twice with PBS, the cells were covered with PBS and fluorescence intensities were measured at excitation/emission wavelengths of 485/530 nm using a SpectraMac M3 microplate reader (Molecular Devices, CA, USA).

Ⅱ-8. Total RNA purification and cDNA synthesis

Hs68 cells cultured in 6-well plates (0.6×10^5 cells/well) were treated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and exposed to UVB (35 mJ/㎠), and then incubated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h in serum-free medium. The cells were lysed and total RNA was isolated by an RNeasy Plus Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. First strand cDNA was synthesized from 500 ㎍ of

RNA with oligo $(dT)_{18}$ primer using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea). Cycling conditions were for 5 min at 25℃, for 1 h at 42℃, and for 15 min at 70℃.

Ⅱ-9. RT-PCR

The cDNA products were used as PCR templates to amplify its target genes. RT-PCR was performed using AccuPower PCR Premix (Bioneer, Daejeon, Korea). Cycling conditions were as follow; denaturation for 30 sec at 94℃, annealing for 30 sec at 48℃, 55℃, or 60℃, and extension for 30 sec at 72℃ for 30 cycles. The sequences of the specific primers used were as follow: MMP-1 forward 5′-GCATATCGATGCTGCTCTTTC-3′, MMP-1 reverse 5′-GATAACCTGGA-TCCATAGATCGTT-3′, human procollagen type Ⅰ forward 5′-CTCGAGGTGGACACC-ACCCT-3′, human procollagen type Ⅰ reverse 5′-CAGCTGGATGGCCACATCGG-3′, GAPDH forward 5′-TCAGCAATGCATCCTGCACCAC-3′, and GAPDH reverse 5′ -TGCCAGTGAGCTTCCCGTTCAG-3′.

Ⅱ-10. Enzyme-linked immunosorbent assay (ELISA)

Total collagen synthesis in Hs68 cells were measured using procollagen type Ⅰ C-peptide ELA kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Briefly, Hs68 cells cultured in 96-well plates (1×10^5 cells/well) were pretreated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and exposed to UVB (35 mJ/㎠), and then incubated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h in serum-free medium. After 100 ㎕ of antibody-POD conjugate solution was added to appropriate wells, 20 ㎕ of the cell culture medium was transferred into the wells within 5 min and incubated for 3 h at

37℃. The contents were removed by suction and the wells were washed with 400 ㎕ of washing buffer for 4 times. And then 100 ㎕ of substrate solution (TMBZ) was added to each well and incubated for 15 min at RT. Subsequently, 100 ㎕ of stop solution was added to all the wells and absorbance was measured at 450 nm using a microplate reader.

Ⅱ-11. Analysis of collagenase activity

Hs68 cells cultured in 6-well plates (0.6×10^5 cells/well) were pretreated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and exposed to UVB (35 mJ/㎠), then incubated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h in serum-free medium. A supernatant of the medium was used to measure the activity of collagenase. Collagenase was dissolved in Tris-HCl buffer for a initial concentration of 0.2 ㎎/㎖ and the synthetic substrate 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg trifluoroacetate salt was dissolved in 100 mM Tris-HCl buffer (pH 7.5 with 4 mM $CaCl_2$) for concentration of 0.3 ㎎/㎖. Ten ㎕ of the sample was incubated with 25 ㎕ of the substrate and 15 ㎕ of the enzyme in buffer for 30 min at 37℃. The mixtures were added with 50 ㎕ of 6% citric acid and 150 ㎕ of ethylacetate and centrifuged for 1 min at 13000rpm. Negative controls were performed with the enzyme. One hundred ㎕ of supernatant was transferred at 96-well plate and absorbance was measured at 320 nm using a microplate reader.

Ⅲ. RESULTS AND DISCUSSION

Ⅲ-1. Effect of NEP on cell cytotoxicity on Hs68 cells

To confirm that NEP affects on cell cytotoxicity, MTT assay was performed on human dermal fibroblast cells (Hs68) after treatments with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 48 h. As shown in Fig 7, NEP showed no effect on cell viability upto 20 ㎍/㎖ (Fig. 7). In addition, NEP exhibited no significant cytotoxic effects on Hs68 cells irradiated with UVB (35 mJ/㎠). These results suggest that NEP has no cytotoxic effect on skin cells.

Ⅲ-2. Activation of MAP kinase signaling pathway by NEP

It has been known that MAP kinases (ERK, p38, and JNK) regulate the induction of autophagy and especially the activated ERK can lead the maturation of autophagic vacuoles (Martin *et al.*, 2006). The activation of MEK/ERK can also modulate Beclin-1 expression by inhibiting mTOR1 and/or mTOR2 (Wang *et al.*, 2009). Although there is a dispute over p38 regulates autophagy (Webber *et al.*, 2010), the autophagy activation by p38 results in the increases of Atg proteins, including Beclin-1 and Atg5. In addition, the increased phosphorylation of p38 is related to the induction of autophagy and ER stress-induced Beclin-1 expression (Kim *et al.*, 2010). JNK also induces autophagy by its phosphorylation activity to Bcl-2, which releases Beclin-1 (Wei *et al.*, 2008) and can regulate the expression of *Beclin*-1 gene through the phosphorylation of c-Jun (Bhui *et al.*, 2010). In this study, the activation ability of NEP to MAP kinase signaling pathway was investigated in Hs68 cells using Western blottings. When the cells were treated with 5 ㎍/㎖ of NEP for 15 min at 37℃, the protein expression levels of p-ERK, p-p38, and p-JNK

were increased to approximately 1.5-, 1.6-, and 1.6-folds, respectively, compared to that of non-treated cells (Fig. 8). These results suggest that NEP can activate MAP kinase signaling pathway.

III-3. Activation of autophagy system by NEP

To determine the activation of autophagy system by NEP, the protein expression levels of autophagy factors were analyzed in Hs68 cells treated with 5 ㎍/㎖ of NEP for 30 min using Western blottings. As shown in Fig. 9, the protein levels of p-mTOR and p-p70S6K were decreased to about 0.6- and 0.5-folds in the presence of NEP (5 ㎍/㎖), compared to that of non-treated control group. In addition, the protein expression levels of Beclin-1, Atg12, and LC3 were elevated to about 1.2-, 1.5-, and 2.1-folds in NEP-treated cells. These results suggest that NEP can lead the activation of autophagy system through the stimulation of MAP signaling pathway.

III-4. Formation of autophagosomes by NEP

The formation of autophagosome is controlled by Atgs through Atg12-Atg5 and LC3 complex. As mentioned earlier section, LC3 is commonly used as a marker of autophagosomes in immunocytochemistry (Liou *et al.*, 1997). To confirm the activation ability of NEP to autophagy system, immunostainings were performed with Alexa 488-conjugated anti-LC3 anti-body and the autophagosomes formed were observed with a confocal microscope (Fig. 10). When the Hs68 cells that were non-exposed or irradiated to UVB (35 mJ/㎠) were incubated with 5 ㎍/㎖ of NEP for 30 min, the autophagosomes could be clearly observed in cytosol (Fig. 10). These results show that NEP can actually activate autophagy system.

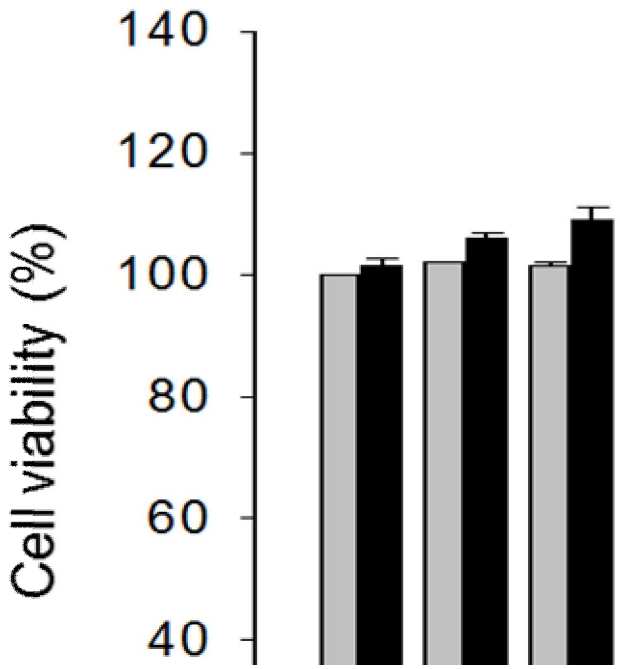

Fig. 7. Effect of NEP on cell cytotoxicity on Hs68 cells. Hs68 cells were cultured in 96-well plates and pretreated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) as indicated for 24 h in serum-free medium. After UVB irradiation (35 mJ/㎠), the cells were post-treated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h in serum-free medium. Cell viability was measured by MTT assay. The optical density value of control was normalized to 100%.

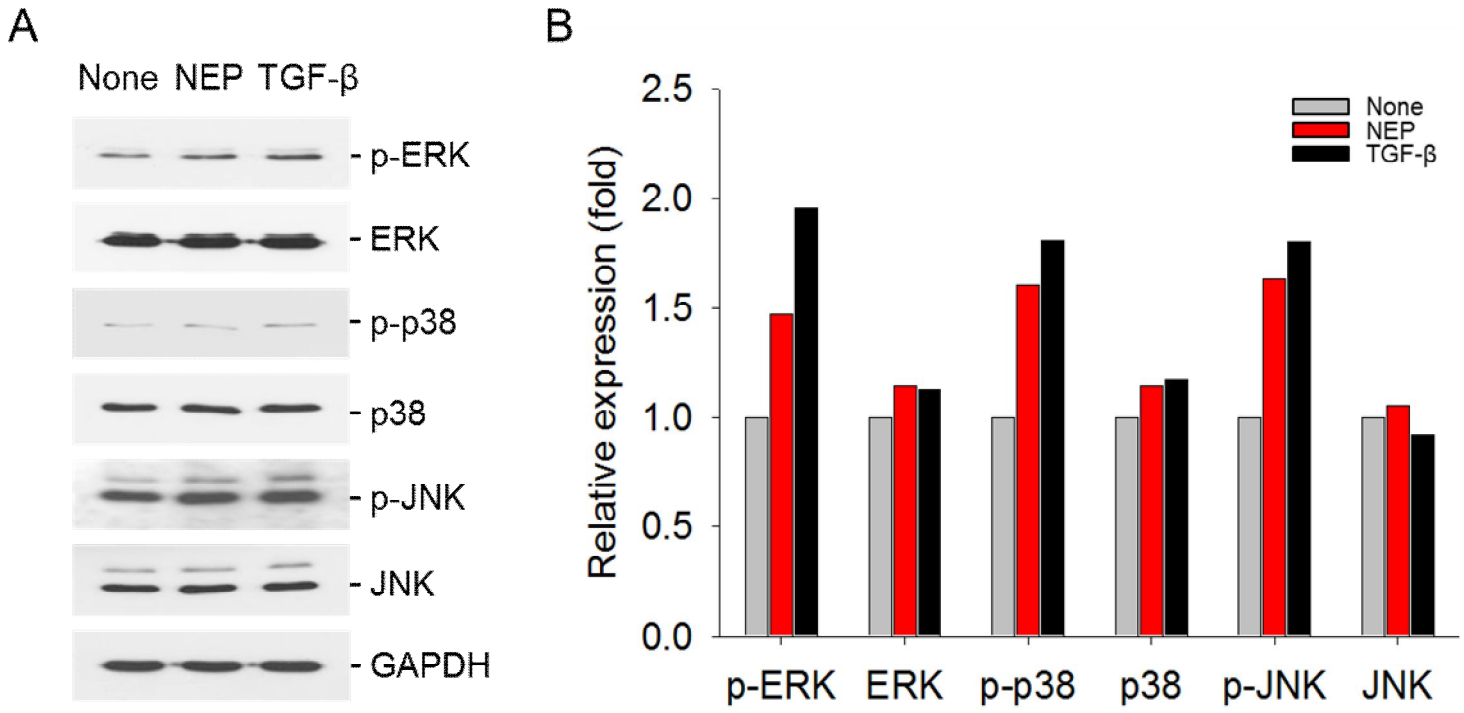

Fig. 8. Activation of MAP kinase signaling by NEP in Hs68 cells. (A) The cells were treated with NEP (5 ㎍/㎖) for 15 min. Cell lysates were prepared and Western blottings were performed using antibodies against p-ERK, ERK, p-p38, p38, p-JNK and JNK (None, negative control; TGF-β (10 ng/㎖), positive control). (B) The relative expressions in fold, which were quantified by densitometry analysis of bands and normalized to GAPDH.

III-5. Inhibitory ability of NEP to the formation of intracellular ROS

Reactive oxygen species (ROS) have been identified as signaling molecules acting in various pathways and also can regulates autophagy (Azad *et al.*, 2009). Human skin exposed to solar ultra-violet radiation (UVR) dramatically increases ROS production/oxidative stresses. It is necessary to understand the characteristics of human skin and how chronological (intrinsic) aging and photo-aging (extrinsic aging) occur via the impact of ROS production (Lephart *et al.*, 2016). To analyze that NEP can inhibit the formation of ROS in Hs68 cells exposed or non-irradiated to UVB, DCF-DA assay was performed (Fig. 11). When the Hs68 cells that were non-exposed or irradiated to UVB (35 mJ/㎠) were incubated with 5 ㎍/㎖ of NEP for 0.5 h or 48 h and then treated with DCFH-DA (20 μM) for 30 min, the production levels of ROS were increased to about 4.1% in UVB-induced cells, while it was decreased to about 14.1% in NEP-treated cells (Fig. 11). These results suggest that NEP can reduce the formation of ROS.

III-6. Effect of NEP on aging factors in Hs68 cells

MAP kinases (ERK, p38, and JNK) signaling pathway control cell growth and differentiation (Chang *et al.*, 2001). The cellular stresses such as heat, UV-light, and inflammatory cytokines involve in the activation of NF-κB and MAP kinases (Wang *et al.*, 2006). Notably, UVB light induces the formation of ROS that evokes aging by activating MAP kinase signaling pathway. To investigate the effects of NEP on aging factors by modulating signaling pathway, Western blottings were performed using antibodies against ERK, p38, and JNK. When the cells were pretreated with NEP (5 ㎍/㎖) for 24 h, exposed to UVB light (35 mJ/㎠), and finally

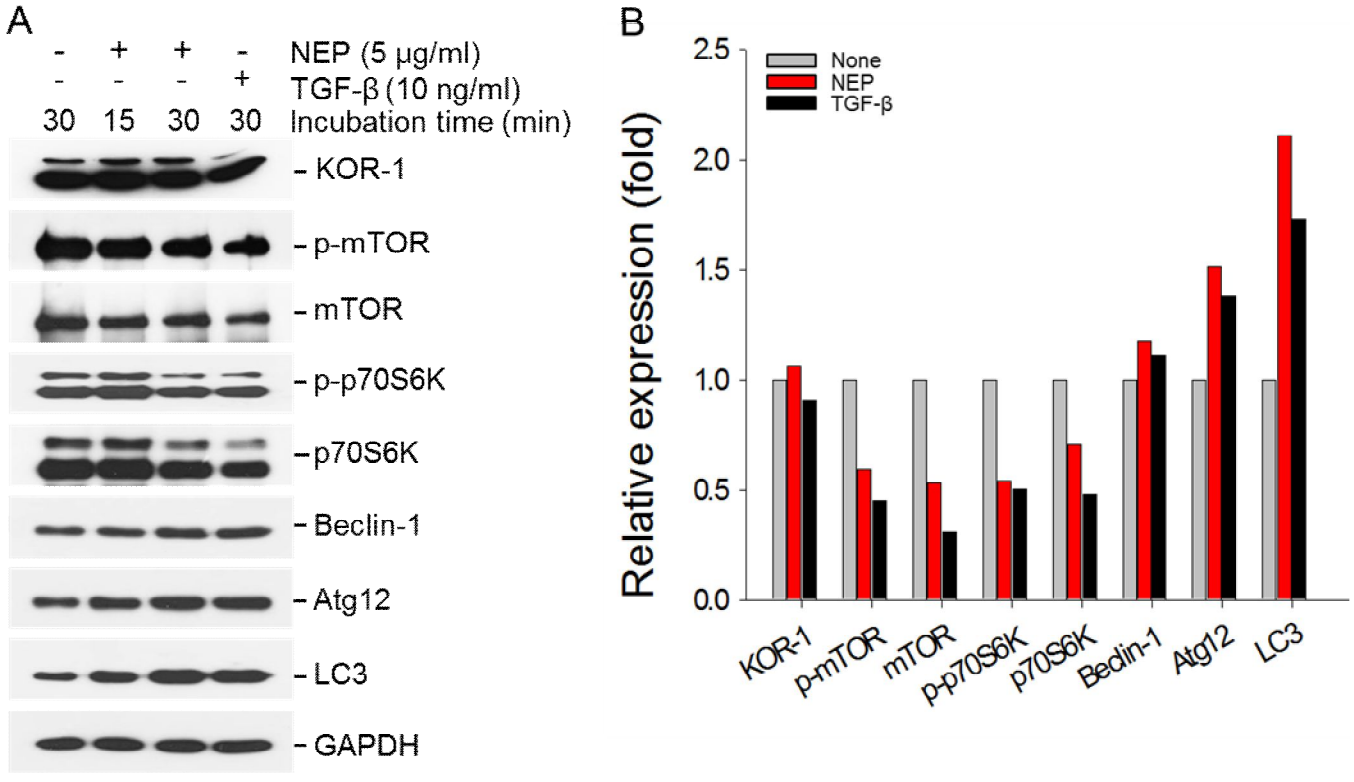

Fig. 9. Activation of autophagy system by NEP in Hs68 cells. (A) The cells were treated with NEP (5 ㎍/㎖) and TGF-β (10 ng/㎖) as a positive control and incubated at 15 min or 30 min. Cell lysates were prepared and Western blottings were performed with antibodies against KOR-1, p-mTOR, mTOR, p-p70S6K, p70S6K, beclin-1, Atg12 and LC3. (B) The relative expressions in fold in fold, which were quantified by densitometry analysis and normalized to that of GAPDH.

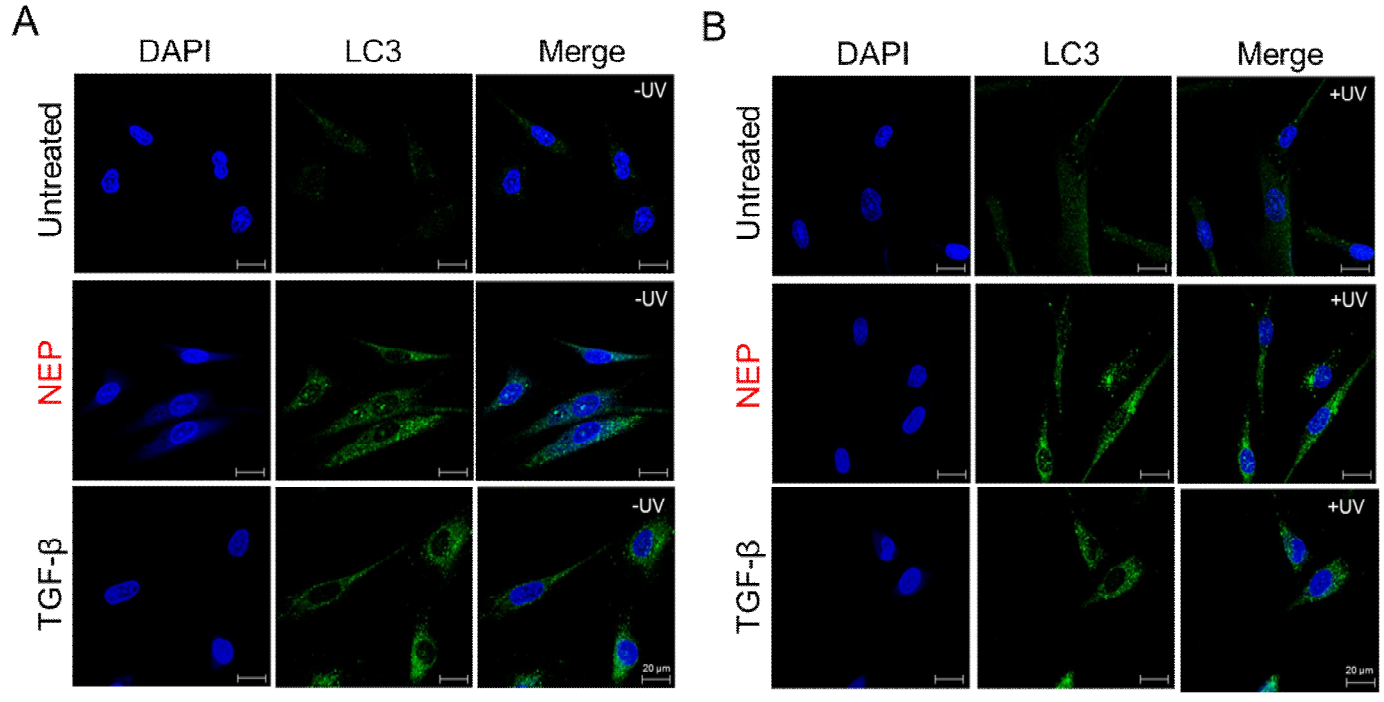

Fig. 10. Confocal immunofluorescence imaging of LC3 in NEP- or TGF-β-treated Hs68 cells. Cells were treated with NEP (5 ㎍/㎖) or TGF-β (10 ng/㎖) for 30 min in the absence (A) or presence (B) of UVB irradiation (35 mJ/㎠), and examined under the Alexa 488 fluorescence for assessment of autophagosomal marker protein LC3 in fixed cells. Confocal images were obtained with a ZEISS LSM510 confocal microscope.

post-treated with the same concentration of NEP for 24 h, the production levels of p-ERK, p-p38, and p-JNK were increased to an average of 22.3-folds in UVB-irradiated cells, whereas their levels were decreased to an average of 6.9-folds in NEP-treated cells. These results suggest that NEP can inhibit the protein expression levels of active pro-aging factors, including p-ERK, p-p38, and p-JNK, delaying photoaging.

III-7. Reduction of collagenase activity by NEP through its suppressive role on the transcription levels of *MMP*-1 gene in UVB-treated cells

UVB irradiation causes skin damages, resulting in photoaging. It has been reported that UVB irradiation can up-regulate MMP-1 expression, which is related to the cleavage of skin matrix proteins such as collagen in the dermal fibroblasts (Koch-Paiz *et al.*, 2004). To examine that NEP can down-regulate the transcription levels of *MMP*-1 gene, through which it show an effect to reduce the collagenase activity in Hs68 cells exposed to UVB light, RT-PCR and collagenase activity assay were performed. When the cells were pre-treated with NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h, exposed to UVB (35 mJ/㎠), and then post-treated with the same concentrations of NEP for 24 h, the transcription level of *MMP*-1 gene was significantly decreased to an average of 18.7%, compared to that of UVB-irradiated control group (Figs. 13A and B). As expected, the collagenase activity also decreased to an average of 16.4% under the same experimental conditions above (Fig. 13C). These results suggest that NEP can prevent UVB-induced extracellular matrix damage by decreasing the collagenase activity.

III-8. NEP-mediated increase of the transcription level of *procollagen type* Ⅰ gene in UVB-irradited cells

It is well known that UVB irradiation decreases the expression of procollagen type Ⅰ (Koch-Paiz *et al.*, 2004). Therefore, the effect of NEP on the expression aspect of procollagen type Ⅰ was examined in the levels of transcription and translation using RT-PCR and ELISA in UVB-irradiated Hs68 cells. As shown in Fig. 14, the transcription levels of *procollagen type* Ⅰ gene were declined to about 22.8% after UVB irradiation. However, the increased transcription of the gene were down to about 7.5% in the presence of 5 ㎍/㎖ of NEP, compared with UVB exposed cell (Fig. 14A and B). In addition, UVB light could suppress the production of the procollagen to about 19.9%. However, NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) could increase its production to an average of 12.5% in UVB-irradiated Hs68 cells (Fig. 14C). These results suggest that NEP can alleviate the UVB-induced extracellular matrix damage, since it shows a great stimulatory effect (approximately 32.4%) on procollagen production in UVB-exposed cells.

Collectively, all results obtained by this study showed that 1) NEP can induce autophagy system through the activation of MAP kinase signaling pathway; 2) NEP-mediated autophagy activation can reduce the formation of intracellular ROS; 3) NEP can decrease the production of aging factors, including ERK, p38, and JNK; 4) NEP can reduce collagenase activity through by its suppressive role on the transcription level of *MMP*-1 gene, and finally 5) NEP can mediate the increase of the transcription level of *procollagen type* Ⅰ gene. On the basis of these results, it could be postulated that NEP can be used for a potential anti-skin photoaging agent (Fig. 15).

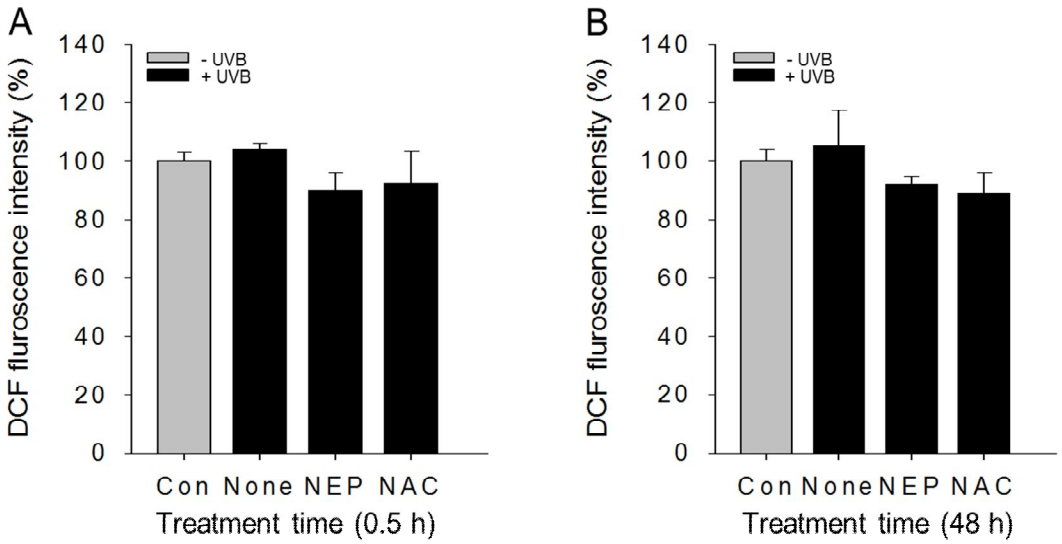

Fig. 11. NEP inhibits the formation of intracellular ROS. Hs68 cells were cultured in 48-well plates and treated with NEP (5 ㎍/㎖) for 0.5 h (A) or 48 h (B). To measure the ROS level the cells treated were incubated with DCFH-DA (20 μM, freshly diluted in serum-free media) for 30 min at 37℃ and the ROS level was assayed using a fluorescence plate reader. NAC (2 mM) was used as positive control.

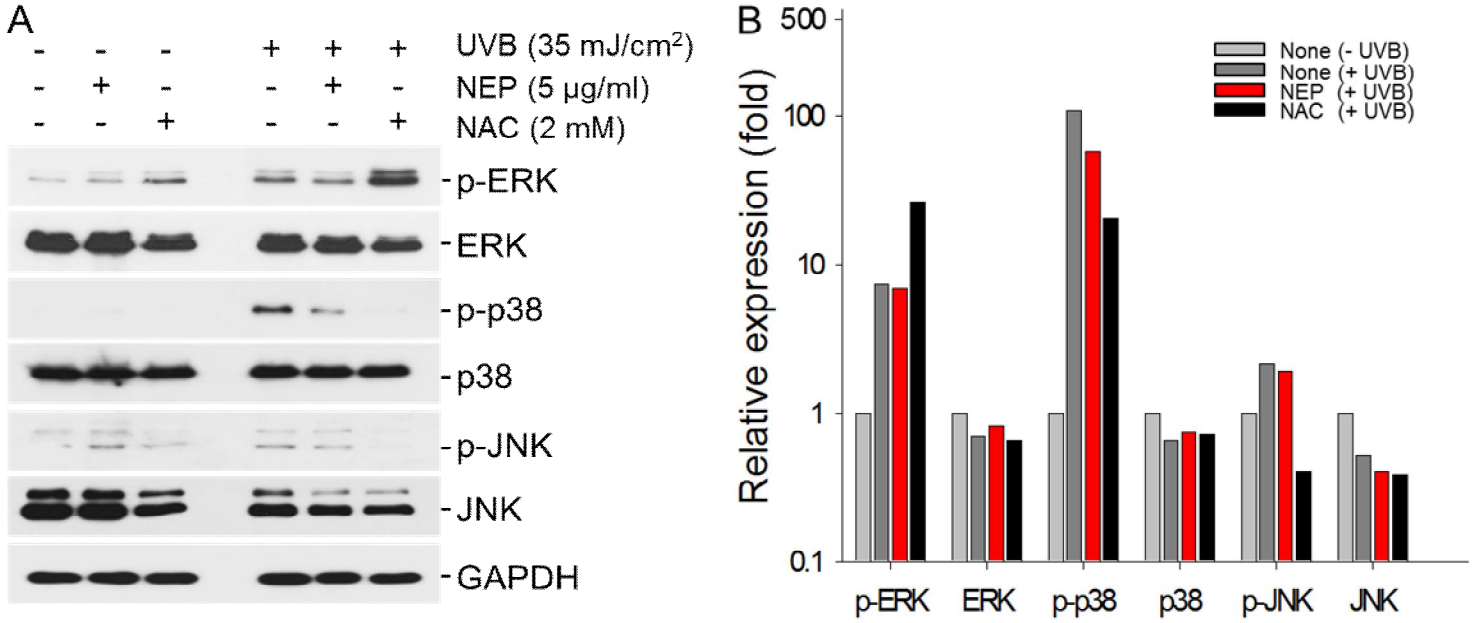

Fig. 12. Effect of NEP on aging factors in Hs68 cells. (A) The cells were pretreated with NEP (5 ㎍/㎖) for 24 h and then exposed to UVB irradiation (35 mJ/㎠). After the exposure, the cells were post-treated with NEP (5 ㎍/㎖) for 24 h and then the cell extracts were prepared and Western blottings were performed with antibodies raised against p-ERK, ERK, p-p38, p38, p-JNK, and JNK. NAC (2 mM) was used as positive control. (B) The relative expressions in fold level, which were quantified by densitometry analysis by normalizing with that of GAPDH.

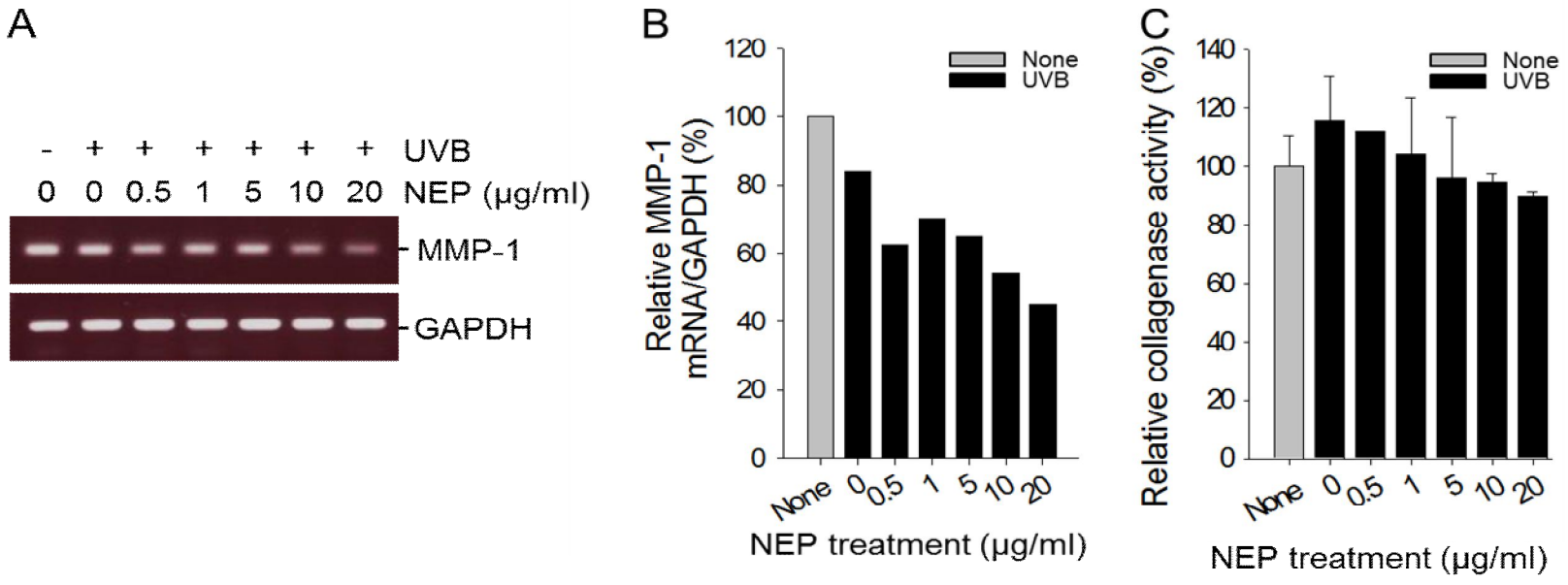

Fig. 13. NEP can inhibit the transcription level of MMP-1, leading the reduction of collagenase activity in UVB-treated cells. (A) Hs68 cells were pretreated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and then exposed to UVB irradiation (35 mJ/㎠). The cells were then post-treated with NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and total cellular RNAs were extracted and the amounts of MMP-1 mRNA expressed were determined by RT-PCR. (B) The relative expression levels were quantified by densitometry analysis of bands, which were normalized to that value of GAPDH. (C) Collagenase activity was measured with culture media using microplate reader.

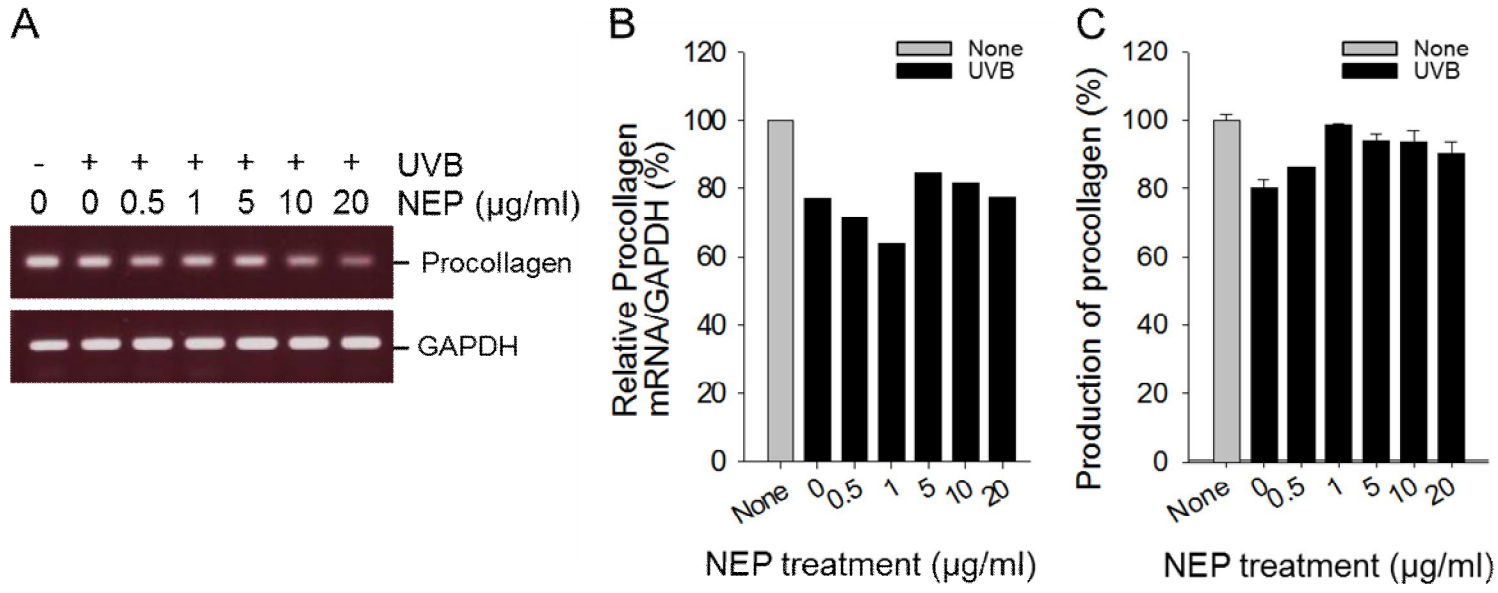

Fig. 14. NEP can increase the transcription level of procollagen type Ⅰ and the production of procollagen type Ⅰ in UVB-induced cells. (A) Hs68 cells were pretreated with various concentrations of NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and exposed to UVB irradiation (35 mJ/㎠). The cells were post-treated with NEP (0.5, 1, 5, 10, and 20 ㎍/㎖) for 24 h and total cellular RNAs were extracted and the amounts of procollagen mRNAs were determined by RT-PCR. (B) The relative expression levels were quantified by densitometry analysis of bands, which were normalized to that value of GAPDH. (C) Procollagen productions were measured with culture media by ELISA.

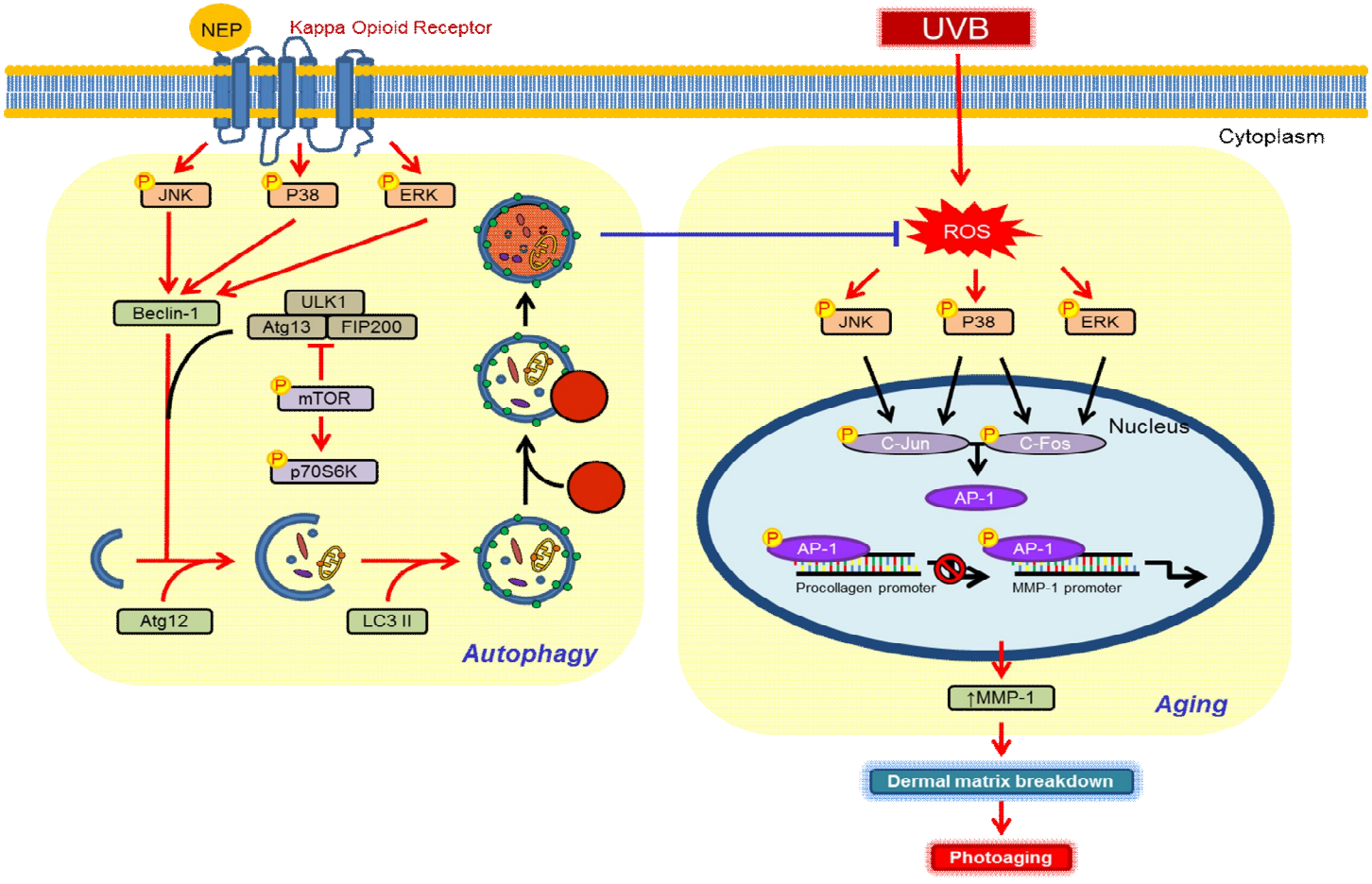

Fig. 15. NEP can induce the activation of autophagy system and lead anti-photoaging through by decreasing the formation of ROS.

Ⅳ. 초 록

인간 피부 섬유아세포에서 항노화 및 autophagy 활성화에 미치는 네오엔돌핀의 영향에 관한 연구

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피부노화(skin aging)란 피부의 구조적 및 기능적 특성이 노화가 진행됨에 따라 퇴행하는 현상을 말한다. 특히 자외선(UV-light)에 의한 광노화(photoaging)는 피부노화에 가장 심대한 영향을 미치는 요인 중 하나이다. 자외선은 막 단백질의 분해, 산화적 스트레스 및 염증 반응 등을 유발하기 때문이다. 세포에서 기능을 다한 세포소기관들 또는 단백질들을 제거하는 과정인 autophagy(자가소화작용 또는 자식작용이라고도 함)는 세포의 노화 및 분화과정에서도 중요한 역할을 하는 것으로 알려져 있다. 또한 최근에는 항노화 및 수명연장에도 중요한 역할을 할 것으로 예측되고 있다. 알파-네오엔돌핀(α-neoendorphin, NEP)은 neuropeptides의 일종으로 10개의 아미노산(H_2N-YGGFLRK-YPK-COOH)으로 이루어져 있으며, kappa opioid receptor를 활성화시켜 세포 내로 신호를 전달한다. NEP는 또한 항노화(anti-aging) 및 항주름(anti-wrinkling)에 효과가 있는 것으로 알려져 있지만 그 작용기작에 대해서는 아직 알려진 바가 없다. 따라서 본 연구에서는 자외선 B(ultraviolet B, UVB)를 조사한 사람의 피부 섬유아세포(Hs68)에서 NEP에 의한 autophagy 활성화 및 항노화 기작을 분석하였다. Western blotting으로 분석한 결과, NEP(5 ㎍/㎖)를 15분간 처리한 Hs68 세포에서 ERK, p38 및 JNK 단백질의 발현양은 모두 증가하는 반면, autophagy의 음성조절자로 알려진 mTOR의 발현양은 감소함을 확인하였다. 또한, Beclin-1, Atg12와 LC3 등과 같은 autophagy-연관 단백질들의 발현도 NEP(5 ㎍/㎖)를 30분간 처리하였을 때 증가함을 확인하였다. 이러한

NEP의 autophagy 활성화능은 autophagosome들을 confocal 현미경으로 관찰함으로써 재확인할 수 있었다. UVB(35 mJ/㎠)를 조사하고 NEP(5 ㎍/㎖)를 30분간 처리한 Hs68 세포들은 실제로 autophagosome들을 갖고 있었다. 한편, NEP는 UVB에 의해 증가된 활성산소(reactive oxygen species, ROS) 생성을 감소시킨다는 사실을 UVB(35 mJ/㎠)를 조사하고 NEP(5 ㎍/㎖)를 처리한 Hs68 세포에서 2′,7′-dichlorofluorescin diacetate(DCFH-DA)로부터 발생하는 형광강도를 측정함으로써 확인하였다. 또한 Hs68 세포에 24시간 동안 NEP(5 ㎍/㎖)를 전처리(pre-treatment)한 후, UVB(35 mJ/㎠)를 조사하고, 동량의 NEP를 24시간 처리한 다음, Western blotting으로 pro-aging factor들(ERK, p38, 및 JNK 등)의 발현량을 분석한 결과, 이들 모두의 발현이 NEP에 의해 평균 6.9% 감소됨을 확인하였다. 또한 동일한 실험조건 하에서 MMP-1 유전자의 전사율은 약 46% 감소되었고, procollagen type I의 전사율은 약 10% 증가됨을 RT-PCR을 통해 확인하였다. Collagenase의 합성기질(synthetic substrate; 4-phenylazobenzyloxy-carbonyl-Pro-Leu-Gly-Pro-Arg trifluoroacetic acid)을 이용하여 NEP를 처리한 세포에서 collagenase 활성을 측정한 결과, collagenase 활성이 평균 35.1% 감소됨을 확인하였으며, ELISA를 이용하여 procollagen의 생성량을 측정한 결과, 평균 32.4%가 증가되었음을 확인하였다. 이러한 결과를 종합하여 볼 때, NEP는 피부세포의 autophagy 시스템을 활성화시키고, 세포 내 유해산소 ROS를 줄이며, MMP-1의 발현 및 활성 억제를 통해 procollagen 생성량을 증가시킴으로서 UVB에 의한 광노화를 억제하는 것으로 판단된다. 따라서, NEP은 피부세포의 항상성에 중요한 역할을 하는 autophagy를 활성화시켜 항노화 및 주름개선에 효과적인 약물일 가능성을 시사한다.

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