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Research Article

The potential inhibitory effect of ginsenoside Rh2 on mitophagy in UV-irradiated human dermal fibroblasts

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ABSTRACT

Background: In addition to its use as a health food, ginseng is used in cosmetics and shampoo because of its extensive health benefits. The ginsenoside, Rh2, is a component of ginseng that inhibits tumor cell proliferation and differentiation, promotes insulin secretion, improves insulin sensitivity, and shows antioxidant effects.

Methods: The effects of Rh2 on cell survival, extracellular matrix (ECM) protein expression, and cell differentiation were examined. The antioxidant effects of Rh2 in UV-irradiated normal human dermal fibroblast (NHDF) cells were also examined. The effects of Rh2 on mitochondrial function, morphology, and mitophagy were investigated in UV-irradiated NHDF cells.

Results: Rh2 treatment promoted the proliferation of NHDF cells. Additionally, Rh2 increased the expression levels of ECM proteins and growth-associated immediate-early genes in ultraviolet (UV)-irradiated NHDF cells. Rh2 also affected antioxidant protein expression and increased total antioxidant capacity. Furthermore, treatment with Rh2 ameliorated the changes in mitochondrial morphology, induced the recovery of mitochondrial function, and inhibited the initiation of mitophagy in UV-irradiated NHDF cells.

Conclusion: Rh2 inhibits mitophagy and reinstates mitochondrial ATP production and membrane potential in NHDF cells damaged by UV exposure, leading to the recovery of ECM, cell proliferation, and antioxidant capacity.

1. Introduction

Exposure to ultraviolet (UV) radiation has numerous adverse health effects, including the induction of inflammation, skin aging, and cancer [1,2]. Sunlight is the main source of UV, and exposure is increased by participation in outdoor recreational activities [3–5]. The skin is one of the largest organs in the human body, constituting approximately 16% of the total body mass; it consists of two basic layers (epidermis and dermis). Because it is in direct contact with the environment, the epidermis responds first to environmental factors, such as infectious pathogens, chemicals, and UV [6–8].

UV exposure produces reactive oxygen species (ROS), which are unstable free radicals that have adverse effects on health [9–11]. ROS produced in greater levels than can be managed by the body's intrinsic antioxidant defense mechanisms can cause oxidative stress, resulting in cell damage, apoptosis, and cell death [12–14]. Mitochondria, organelles closely related to ROS production, are responsible for ATP production because of oxidative phosphorylation [15]. In addition, mitochondria have essential roles in initiating apoptosis and autophagy. Excessive ROS stress induces mitophagy, a selective autophagy process in mitochondria, which is considered the main mechanism underlying the removal of damaged or unnecessary mitochondria [16–18].

There are several types of ginsenosides; of these, Rh2 is a rare panaxidol with potential therapeutic effects in cancers (e.g., breast, leukemia, prostate, and pancreatic). Additionally, Rh2 shows

Abbreviations: NHDF, Normal human dermal fibroblast; ECM, extracellular matrix.

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anticancer activity by inhibiting the cell cycle in cancer cells; it also induces apoptosis [19–22]. Rh2 is reportedly effective against UV-damaged skin cells and exhibits anticancer effects [23]. However, the relationship between the mitochondrial components responsible for ROS generation and the effects of Rh2 remains unclear.

This study examined the functional roles and effects of Rh2 on mitochondria, which are responsible for the regenerative capacity of skin cells damaged by UV and ROS generation. Rh2 restored the expression of UV-damaged skin-related genes; it also restored mitochondrial function. Furthermore, excessive ROS generation was reduced and mitophagy was suppressed in UV-exposure normal human dermal fibroblast (NHDF) cells.

2. Materials and Methods

2.1. Rh2 material

The ginsenoside Rh2 (≥97% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and transfection

Normal Human Primary Dermal Fibroblasts (NHDF) cells were purchased from ATCC (PCS-201012, Manassas, VA, USA). NHDF cells were maintained in culture in Dulbecco's Modified Eagle's Media containing 10% fetal bovine serum and 1% antibiotic. NHDF cells were transiently transfected with pDsREd2-Mito and GFP-LC3 plasmid by using JetPEI reagents (poly plus transfection, NY, USA).

2.3. Analysis for cell viability

Cell viability assay were performed as previously described [24]. The cells were treated with Rh2 at dose of 1, 10, 50 μM Rh2 extract for 24 h.

2.4. Real-time assay for cell proliferation

Cell proliferation was measured using the xCELLigence RTCA DP system (Roche Diagnostics, Indianapolis, IN, USA), which monitors cells in real-time. That is, cells were plated in well of E-Plate 16 (for proliferation; NHDF 1×10^4) and incubated for indicated times.

2.5. Wound-healing assay

To perform cellular wound healing assays, monolayers of cells were scraped using a 10 μl pipette tip. After 24 h of irradiating (80 mJ/cm^2) the cells with UV with a UV crosslinker (Vilber Lourmat, Collegien, France), the cells were un treated or treated with Rh2 extract at a concentration of 1, 10, or 50 μM for 24 h. Thereafter, the wound site was photographed using a microscope.

2.6. Antibodies and western blotting analysis

Western blot analysis was performed as previously describes [24–26]. Briefly, cell were placed on ice and extracted with lysis buffer including 1% v/v Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 25 mM sodium fluoride, 120 mM NaCl, 0.1 mM sodium orthovanadate, 40 mM β-glycerol phosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 2 μM microcystin-LR. Lysates were centrifuged for 30 min at 13,000 rpm. The lysates were loaded by 7.5–12.5% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). The membranes were blocked for 1 h in 1 X tri-buffered saline buffer (TBS; 2.7 mM KCl, 140 mM NaCl, 250 mM Tris- HCl, pH 7.4), including 5% skim milk and 0.1% Tween-20, followed by an overnight incubation with each protein's 1st antibodies diluted 1000-fold or 2000-fold at 4 °C. The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Komabiotech), diluted 2000-fold or 5000-fold in the 1X TBSt buffer. To detect protein expression, it was visualized by intensified chemiluminescence according to the manufacturer's instructions (Thermo Fisher Scientific).

2.7. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as previously described [25]. The sequences of the primers sued for qRT-PCR were as follows (F: Forward, R: Reverse); human GAPDH: F-5′-TCGACAGTCAGCCGCATCTTCTTT/R-5′-TACGACCAAATCCGTTGACTCCGA; human Collagen F-5′-TCCCCAGCCACAAAGAGTCTA/R-5′-TTTCCACACGTCTCG GTCA/human Elastin; F-5′- CTGCAAAGGCAGCCAAATAC/R-5′- CACCAGGAACTA ACCCAAACT; MMP-2; F-5′- GTGCTGAAGGACACACACTA AAGAAGA/R-5′-TTG CCATCCTTCTCAAAGTTGTAGG; human COL1 A1; F-5′- AGCCAGCAGATCGAGAACAT/R-5′- TCTTGTCCTTGGGGTTCT TG.

2.8. ROS assay

ROS assay was performed as previously described [24]. NHDF cells were plated and incubated at a density of 5×10^5 cells/well in 6-well culture plates for 24 h. Thereafter, cells were irradiated with UV (80 mJ/cm^3) using a UV crosslinker, and then incubated for 24 h untreated or treated with the Rh2 extract.

2.9. Total antioxidant capacity assay

Cells were washed tree times with cold-PBS and then homogenized with 100 μL of 60 mM NaOH. The homogenates were centrifuged at 300 rpm for 15 min, and the supernatant was used for analysis (Cell-biolabs, CA, USA). Uric acid and BHT, known as standards, or samples were dispensed in a 96-well plate, and 180 μL of 1X reaction buffer was added to each well. The concentrations of Uric acid and BHT proceeded the same as the Rh2 concentration. After reading the plate at an absorbance of 490 nm, the reaction was initiated and 50 μL of Copper Ion reagent was added to each well. Then, incubated for 5 min in an orbital shaker. Terminate the reaction by adding 50 μL of 1X stop solution and read the plate with the same absorbance.

2.10. Confocal imaging analysis

NHDF cells were incubated on glass coverslips until about 70% confluency. And NHDF cells were transfected the pDsRed2-Mito and GFP-LC3 plasmid using JetPEI reagents. Confocal analysis was performed as previously described [24,25].

2.11. Measurement of ATP levels in subcellular compartments

ATP produced by mitochondria was measured in the same way as previously described [27]. That is, until the value of luminescence reached the maximum value, it was measured at 5 s intervals using a luminometer. To normalize the expression of luciferase in the transfected cells, the luminescence value of each cell was obtained using a luciferase assay kit (Promega, Madison, WI, USA). This was expressed as a ratio to luminescence measured in cells lysed using the same amount of reagent.

2.12. Measurement of mitochondria membrane potential ($\Delta\Psi m$)

After irradiating the NHDF cells with UV at 80 mJ/cm^3, and untreated or treated Rh2 extract at 1, 10, 50 μM. As a control, NHDF cells not irradiated with UV were treated Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 8 h. Cells were stained with 2 μM JC-1 dye for 30 min to measure the mitochondrial transmembrane potential using flow cytometry. At this time, at least 10,000 cells were analyzed for each sample.

2.13. Statistical analysis

Statistical analysis was performed as previously described [28].

3. Results

3.1. Effect of Rh2 on cell proliferation and wound-healing in NHDF cells

NHDF cells were treated with 1, 10, 50, 100, 200, or 400 μM Rh2 extract for 24 h. Treatment with 50 μM Rh2 extract did not exhibit cytotoxicity compared with untreated cells, but cytotoxicity was observed at doses >50 μM (Fig. 1A). Therefore, all subsequent experiments were performed at a maximum dose of 50 μM Rh2 extract. To investigate the anti-apoptotic effect of RH2, apoptosis was induced in NHDF cells by exposure to 80 mJ/cm^3 of UV [24]. In vitro cell proliferation assays showed that Rh2 extract increased NHDF cell proliferation in a dose-dependent manner (Fig. 1B). Wound-healing assays were performed by exposing confluent monolayers of NHDF cells to UV irradiation, followed by treatment with Rh2 for 24 h. The wounds have rapidly recovered in a Rh2 concentration-dependent manner (Fig. 1C and D). Taken together, these data suggest that Rh2 has a recovery function in UV-irradiated skin cells.

3.2. Effects of Rh2 on cell development and protection effect against UV exposure in NHDF cells

Protein kinase B (PKB)/AKT, which is known to be activated by growth factors or insulin, plays a central role in this signaling pathway. In addition, it is known to regulate cellular processes such as cell proliferation, angiogenesis, transcription, nutrient metabolism, apoptosis and cell growth [29]. Full activation of PKB/AKT is induced by the phosphorylation of Ser473 and Thr308 [30,31]. A previous study showed that PKB/AKT phosphorylation was induced in mouse epidermal cells that had been exposed to UV irradiation, indicating a relationship between UV exposure and the PKB/AKT pathway [32].

After UV exposure to NHDF cells, the Rh2 extract was incubated for 24 h treated or untreated. Then, the protein extract of NHDF cells was analyzed by Western blot. The results showed that the pERK (T44/T42) and PKB (S473) expression levels were significantly increased by treatment with Rh2 extract, in a dose-dependent manner (Fig. 2A and B). In previous studies, it is known that the growth factor receptors of Src and Ras-MEK-MAPK are also activated by UV exposure. The UV exposure to HaCaT cells, a human keratinocyte cell line, altered the nuclear translocation of the epidermal growth factor receptor (EGFR) and changed the structure of the EGF binding site [33]. This results indicate that the expression of TNFR1, EGFR and pp38 proteins increased by UV exposure was reduced by the Rh2 extract. Additionally, the expression of cleaved caspase 3, a marker of apoptosis, was reduced by Rh2 extract (Fig. 2C and D). Taken together, these observations suggested that Rh2 extract suppresses UV-induced increases in the expression of several growth-related genes.

3.3. Rh2 affects the expression of ECM-related genes in UV exposure NHDF cells

The components of the ECM regulate cellular structure via intercellular communication, cell adhesion, and differentiation; a balanced arrangement of ECM components is important for supporting the skin [34]. Dermal ECM is mainly composed of fibrinous collagen and elastic fibers, which are synthesized by fibroblasts. These ECMs provide elasticity and strength to the skin [35]. The ECM is regulated under normal physiological conditions, but disorders can lead to chronic inflammation and diseases (e.g., cancer) [34].

Collagen, a component of the ECM, is a structural protein primarily found in connective tissues, such as cartilage, bone, tendon, ligament, and skin. As the main component of connective tissue, collagen is the most abundant protein, constituting 25%–35% of the total protein in the body. Collagen combines with amino acids to form a triple helix of long helical fibrils [36–41].

The main function of elastin is to restore the shape of contracted or stretched tissue, which is known as a stretchable protein; similar to collagen, elastin is mainly present in connective tissue. In addition, elastin is present as a structural protein in load-bearing tissues in vertebrates [42–44].

Matrix metalloproteinase (MMPs) are involved in tissue remodeling, such as arthritis and tumor metastasis, as well as in many physiological processes. MMP-3 breaks down collagen, fibronectin, and elastin; it may also activate other MMPs that are important for connective tissue remodeling. This enzyme is known to be mainly involved in tumor initiation and progression of atherosclerosis [45].

We confirmed ECM protein expression in Rh2-treated cells after UV exposure. Our data showed that elastin expression was increased by Rh2 in a dose-dependent manner, whereas MMP-3 levels were decreased (Fig. 3A and B). Notably, the collagen I protein level was not affected by Rh2 extract (Fig. 3A). The MMP-3 protein level increased after UV irradiation compared with the normal state, but it was reduced by Rh2 treatment in a dose-dependent manner after UV irradiation (Fig. 3A and B). Next, we performed real-time quantitative reverse transcription PCR (qRT-PCR) analysis to determine the expression levels of several mRNAs that encode ECM proteins in untreated or treated Rh2 cells after UV exposure. After UV irradiation, the basal expression levels of collagen, elastin, and COL1A1 were decreased, but the effect on elastin expression was ameliorated by Rh2 treatment in a concentration-dependent manner, although the level of expression remained significantly lower than in nonirradiated controls (Fig. 3C and D). There were no significant differences in collagen or COL1A1 mRNA levels between Rh2-treated and untreated cells after UV irradiation (Fig. 3D, F). Whereas, the expression levels of MMP-2 was decreased after treatment of Rh2 (Fig. 3E). The results of mRNA expression analyses were thus similar to the results of protein expression analyses; they suggested that Rh2 treatment would have protective effects in UV-irradiated cells.

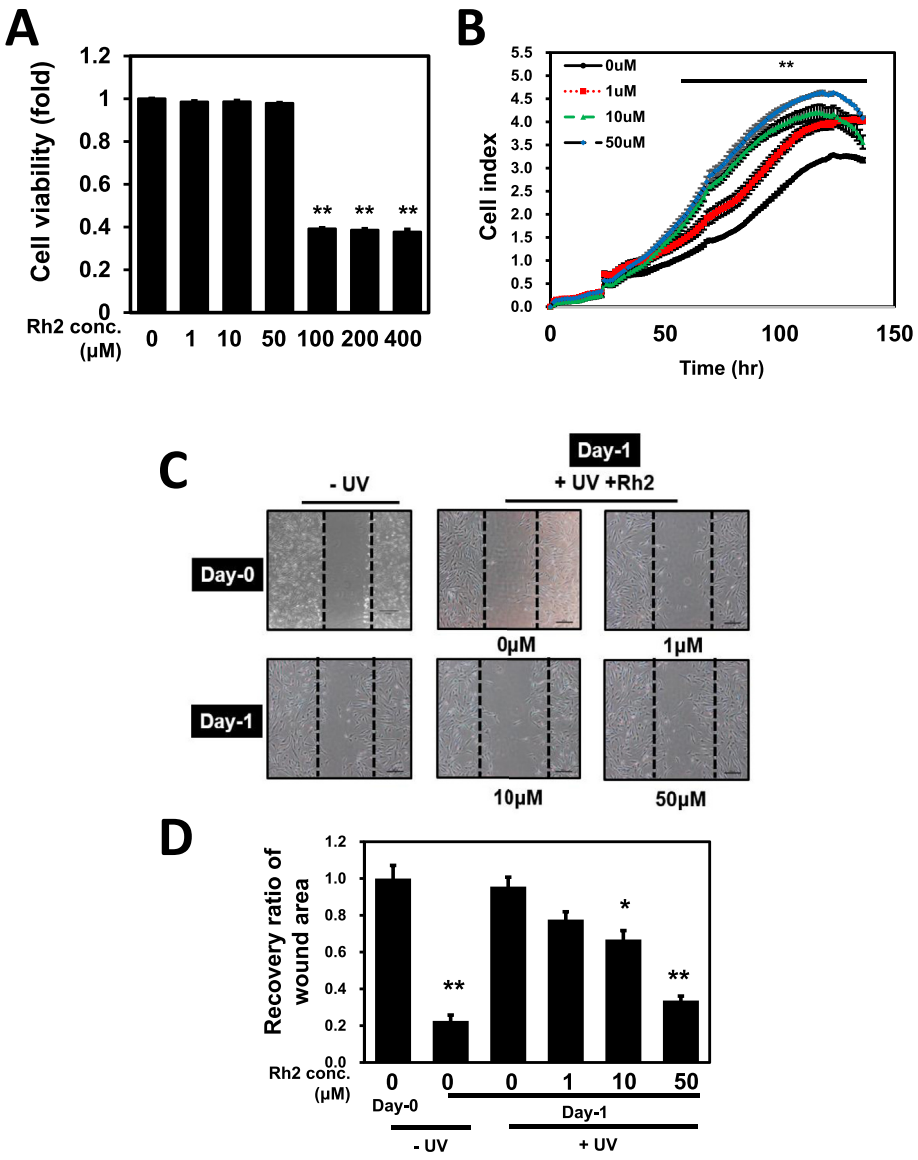

Fig. 1. Cell viability and Cell proliferation assay. (A) Rh2 extract showed no cytotoxicity at 1, 10 or 50 μM in EZ-Cyto assay using NHDF cells. (B) Proliferation of Rh2-treated NHDF cells were measured for 120 h using the xCELLigence system. (C) Cell wound healing assay was performed 24 h on UV-irradiated cells after scratching. UV-irradiated NHDF cells were further treated with Rh2 extract in a dose-dependent manner. (D) Relative proportions of wound sizes. Results are presented as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

3.4. Treatment with Rh2 extract affected antioxidant levels after UV irradiation in NHDF cells

The generation of ROS is triggered by the environment as well as cellular processes, such as aging, apoptosis, and metabolism. Antioxidants protect against UV-induced skin damage. The ROS removal system consists of antioxidants and antioxidant enzymes [46]. In the present study, fluorescence-activated cell sorting (FACS) analysis showed a decrease in ROS production in Rh2-treated cells, compared with untreated controls (Fig. 4A). The antioxidant activity was examined after confirming the decreases in ROS levels by Rh2. The results indicated that the antioxidant activity was increased by Rh2 to an extent similar to the findings in uric acid and BHT-positive controls; the UV-induced reduction of total antioxidant capacity was increased by Rh2 in a dose-dependent manner (Fig. 4B). These results suggest that Rh2 inhibits in skin cells from UV by reducing the production of ROS.

Nuclear factor erythroid 2-related factor 2 (NRF2) increases antioxidant potential and inhibits oxidative stress [47]. In a previous study, the UV irradiation of human corneal endothelial cells induced antioxidant defense mechanisms involving increased expression of NRF2 at both transcriptional and translational levels [47]. Furthermore, NRF2 regulated antioxidant activity by increasing the expression of HO-1 in UV-irradiated cells [48]. Here, the protein expression levels of NRF2, which is related to antioxidant defense, and HO-1, which acts downstream of NRF2, were investigated in UV-irradiated NHDF cells (Fig. 4B). Upon Rh2 treatment, the UV-induced reductions of NRF2 and HO-1 protein

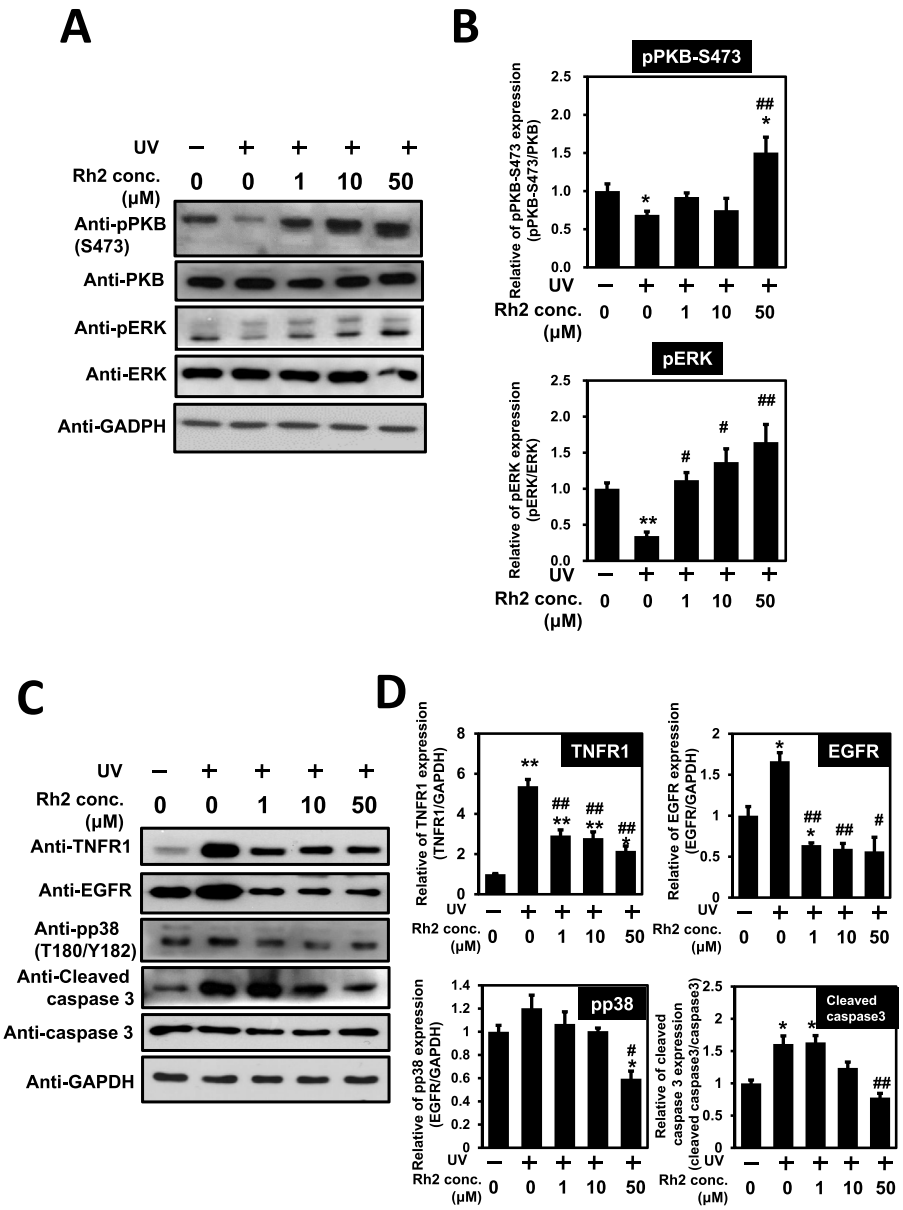

Fig. 2. Expression of proteins involved in cell development and proliferation using Western blotting. (A, C) Proteins extracted from untreated cells or Rh2 extract-treated cells were treated with several antibodies (extracellular signal-regulated kinase (ERK), phosphorylated ERK2, protein kinase (PKB), phosphorylated PKB, tumor necrosis factor receptor 1 (TNFR1), epidermal growth factor receptor (EGRF), analyzed by western blotting using phosphorylated p38, cleaved caspase 3, and caspase 3). (B, D) Antibodies (ERK, pERK, PKB, pPKB, TNFR1, EGFR, and pp38) were quantified by Western blot analysis, as described in the Materials and Methods. The values are means ± SD of three independent experiments. Significant difference from negative control (Non UV irradiated) *, $p < 0.05$ and **, $p < 0.01$. Significant difference from positive control (UV irradiated) $^{\#}$, $p < 0.05$ and $^{\#\#}$, $p < 0.01$.

expression levels were restored to levels similar to the findings in nonirradiated control cells (Fig. 4C and D). These results indicated that treatment with Rh2 increases the expression of RNF2 and HO-1.

3.5. Rh2 restores the morphology and function of mitochondria damaged by UV irradiation in NHDF cells

Mitochondrial fission and fusion occur in various organisms, including yeast, flies, and mammals; they are mediated by guanosine triphosphate (GTPase) [49]. Fission is mediated by the cytoplasmic dynamin family member, DRP1, which forms a helix around mitochondria and cleaves the inner and outer membranes. Mitochondrial fission begins with the division of the inner and outer membranes [50]. In mitochondrial fusion, the outer and inner membranes are induced separately. Fusion between the outer membranes is initiated by the membrane-anchored dynamin family members MFN1 and MFN2. Inner membrane fusion is mediated by a single dynamin family member, Opa1 [51,52]. UV irradiation decreased the expression levels of fusion-related proteins and increased the expression levels of fission-related proteins. However, treatment with Rh2 ameliorated these changes in protein

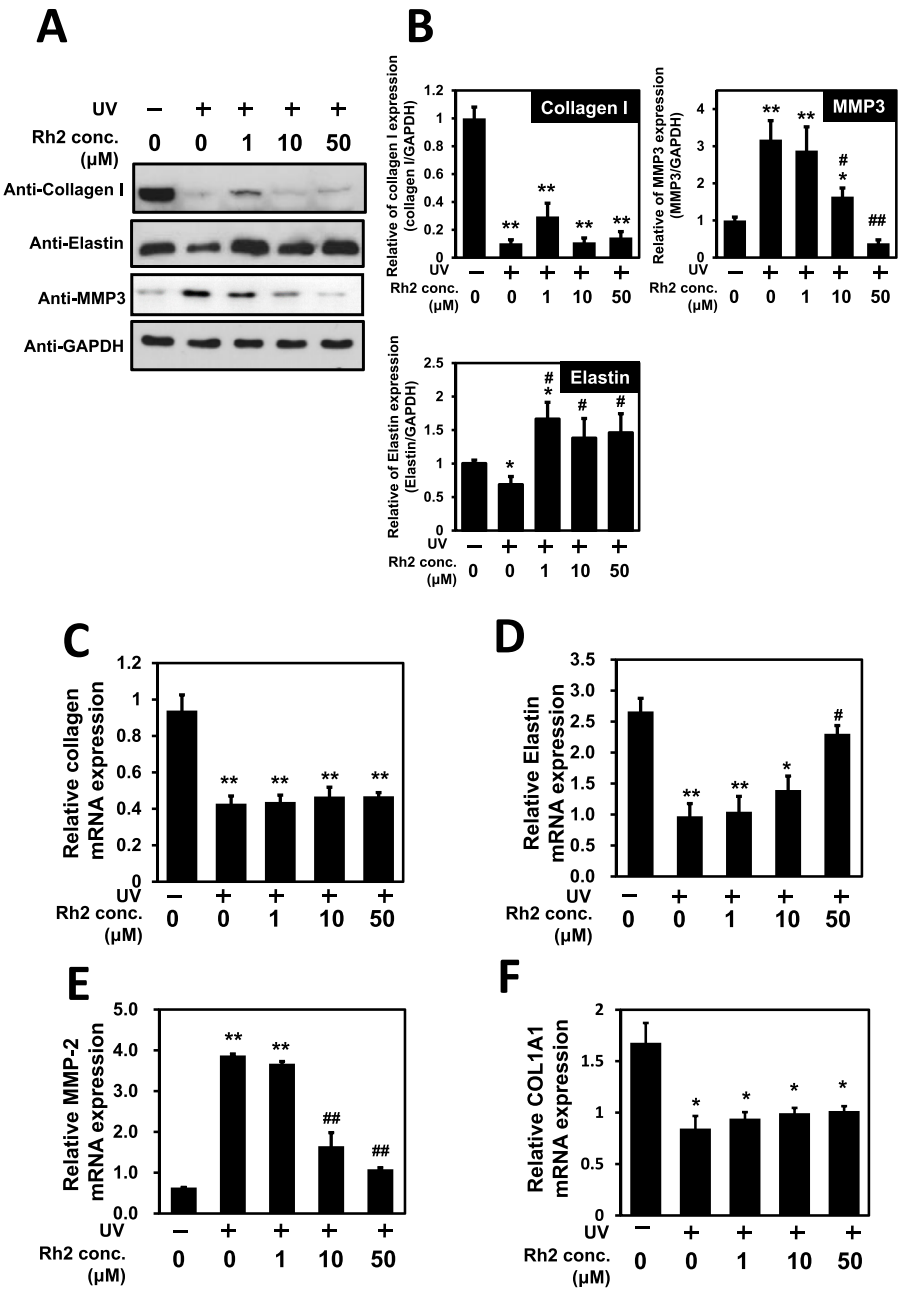

Fig. 3. Expression levels of extracellular matrix mRNA and protein. (A) Total protein levels of ECM (Collagen I, Elastin, and MMP3) in non-treated and Rh2 treated cell on UV irradiation. (B) The immunoblotting were quantified by Western analyses, as described in Methods. Bar heights are means ± S.D. of three independent experiments. Significant difference from negative control (Non UV irradiated) *, $p < 0.05$ and **, $p < 0.01$. Significant difference from positive control (UV irradiated) #, $p < 0.05$ and ##, $p < 0.01$. (C, D, E, F) mRNA expression levels of ECM related genes in non-treated cells and Rh2 treated cell on UV irradiation.

expression. The levels of fusion-related proteins, such as MFN2, was increased in Rh2-treated cells compared with untreated controls. In addition, the levels of the mitochondrial fission-related proteins, pDRP1(S616), Fis1, and MFF, were decreased in Rh2-treated cells (Fig. 5A and B). However, the level of pKRP1 (S637), one of the fiction-related proteins, tended to be increased by Rh2. Confocal microscopy analyses of cells exposed to UV irradiation were performed to examine mitochondrial morphology; the results were similar to the findings after treatment with CCCP, a mitochondrial uncoupler that induces mitochondrial autophagy (mitophagy). The fragmented mitochondria in UV-treated cells changed to a tubular form in cells that had been treated with Rh2 at 10 μM and 50 μM (Fig. 5C). These results show that Rh2 extract affects mitochondrial dynamics. Mitochondrial membrane potential decreased by UV exposure in NHDF cells was increased by Rh2 treatment (Fig. 5E). After UV irradiation, mitochondrial membrane potential and mitochondrial ATP level were significantly increased in Rh2-treated cells, compared with untreated control (Fig. 5D and E). These

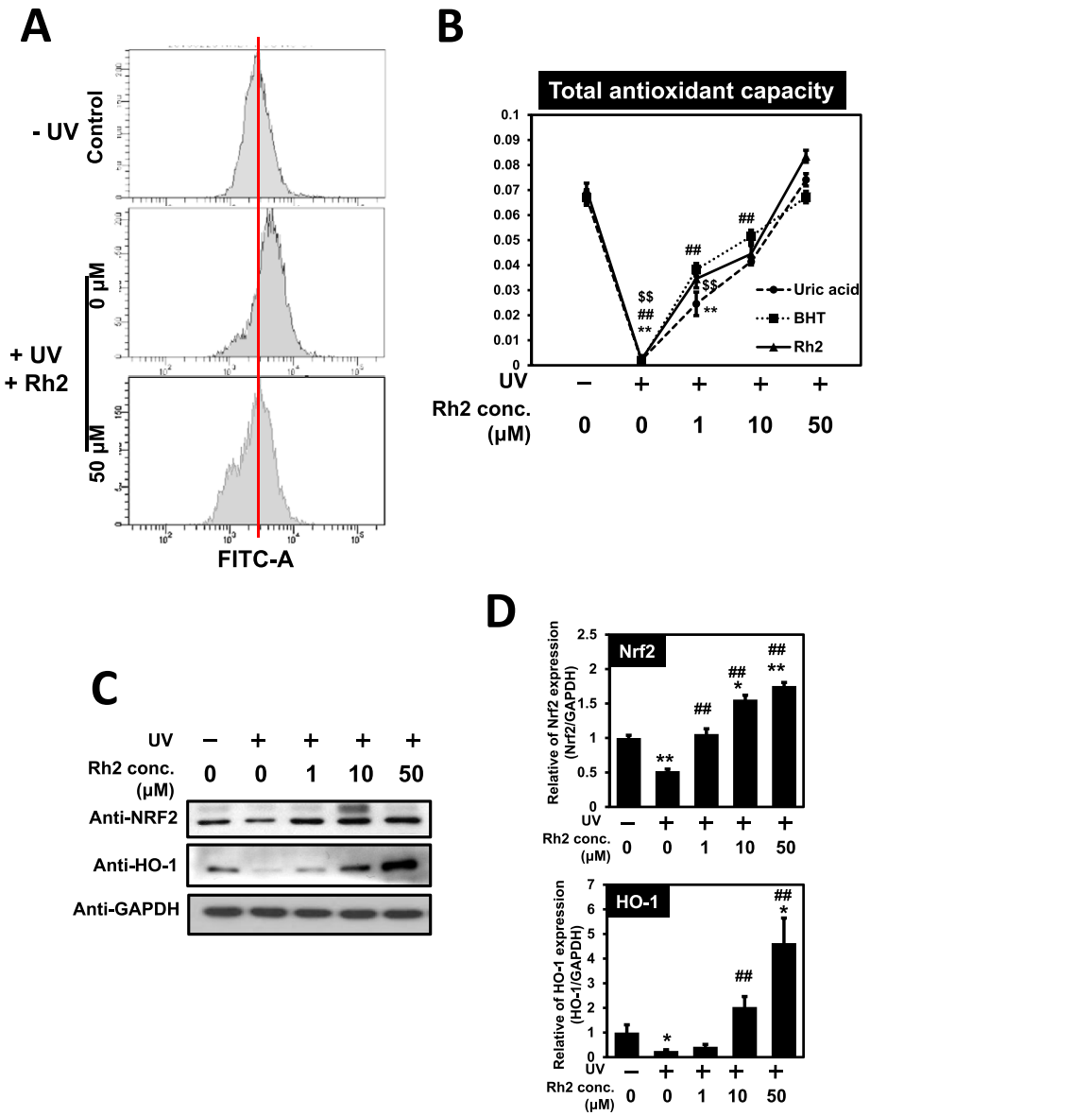

Fig. 4. Rh2 decreases ROS production through antioxidant effects. (A) For ROS measurement of H_2O_2, 2′, 7′-dichlorodehydrofluorosine diacetate (CM-H2DCFDA) was used. (B) Total antioxidant activity was indicated by measuring the absorbance at 490 nm after treatment with the Rh2 extract. Uric acid and BHT (butylated hydroxytoluene) were used as controls. *p < 0.05; **p < 0.01, versus between BHT samples; #p < 0.05, ##p < 0.01, versus between Uric acid samples; $$p < 0.01, versus between Rh2 (C) Protein level of HO-1, NRF2 from UV exposed Rh2 treated samples by western blotting analysis. (D) Graphical representation. Significant difference from negative control (Non UV irradiated) *, p < 0.05 and **, p < 0.01. Significant difference from positive control (UV irradiated) ##, p < 0.01.

observations suggested that Rh2 extract induces mitochondrial fusion and the recovery of mitochondrial function in UV-irradiated dermal fibroblast cells.

3.6. Rh2 inhibits mitophagy induced by UV irradiation

Damaged mitochondria are removed to maintain homeostasis in the body through an autophagic process known as mitophagy, which is mediated by the PINK1-Parkin signaling pathway. PINK1 and parkin physically interact, and the mitochondrial potential of parkin is dependent on PINK1 [53–56]. PINK1 is localized in the intermembrane space or the outer membrane of mitochondria. When mitochondria are damaged and the membrane potential is reduced, PINK1 in the intermembrane space binds to the outer membrane of the mitochondria. Parkin then binds to PINK1 and causes fusion of the lysosome and autophagosome (similar to autophagy), eventually leading to mitochondrial degradation [57].

To assess the *anti*-mitophagy effect of Rh2, NHDF cells were induced to undergo mitophagy by exposure to UV irradiation at a dose of 80 mJ/cm3; time-dependent changes in PINK1 and Parkin protein expression levels were examined by Western blotting (Fig. 6A). Based on the results, subsequent experiments were performed using UV irradiation at a dose of 80 mJ/cm3 for 3 min. Mitophagy induced by UV exposure was inhibited by Rh2

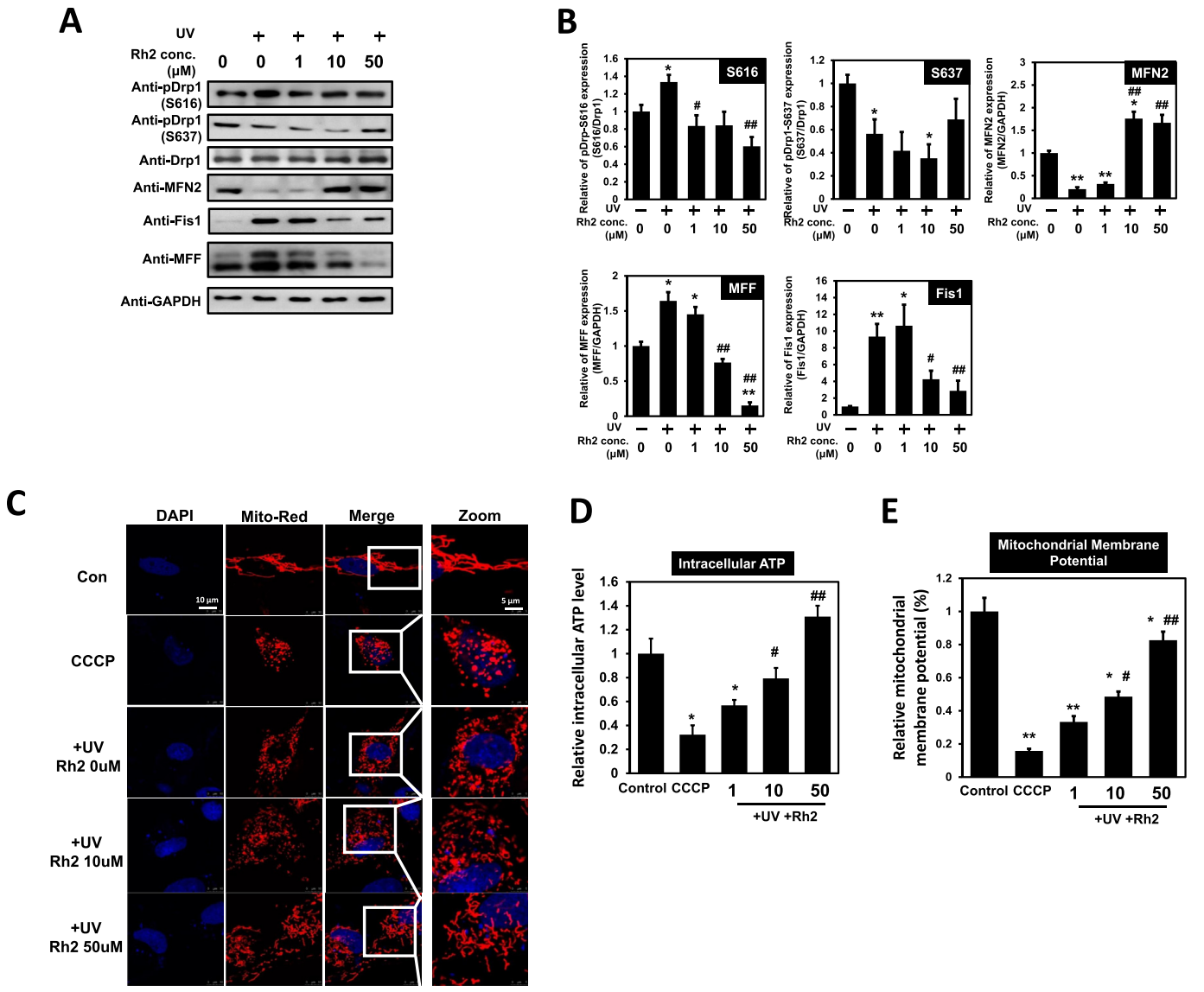

Fig. 5. Changes the mitochondria morphology and function Rh2 treatment after UV irradiation. (A) The expression levels of Fission (Drp1, Fis1, and MFF) and Fusion (Opa1 and MFN2) protein in untreated and treated of Rh2 cell line after UV exposure (B) Graphical representation. Significant difference from negative control (Non UV irradiated) *, $p < 0.05$ and **, $p < 0.01$. Significant difference from positive control (UV irradiated) $^{\#}$, $p < 0.05$ and $^{\#\#}$, $p < 0.01$. (C) Image of Mito-red staining from control samples (non-UV exposure) and UV exposed Rh2 treated samples. Scale bars, 10 μm or 5 μm (D) ATP of mitochondria (E) Mitochondrial membrane potential (ΔΨm; mitochondrial respiratory capacity) from control samples (non-UV exposure) and UV exposed Rh2 treated samples. Significant difference from negative control (Non UV irradiated) *, $p < 0.05$ and **, $p < 0.01$. Significant difference from positive control (UV irradiated) $^{\#}$, $p < 0.05$ and $^{\#\#}$, $p < 0.01$.

treatment in a dose-dependent manner. The UV-induced increases in Parkin and PINK1 expression levels were decreased by Rh2 treatment; the expression of LC3-II, an autophagy marker, was significantly decreased. These results were also confirmed by confocal microscopy; LC3 puncta were also reduced by Rh2 treatment (Fig. 6B and C). These observations suggested that Rh2 inhibits UV-induced mitophagy.

4. Discussion

Ginseng is a widely used herbal medicine; various pharmacologically active ginsenoside components have been reported and are used in cosmetics and health foods. Rh2 is a ginsenoside that has antioxidant effects on human keratinocytes and dermal fibroblasts. To our knowledge, there have been no previous reports of the relationships between UV-damaged mitochondria and Rh2 or the inhibitory effect of Rh2 against mitophagy in dermal fibroblasts. Here, we analyzed various beneficial effects of Rh2 extract in UV-exposed NHDF cells, including inhibitory effects on skin aging, antioxidant effects, the recovery of mitochondrial function, and the inhibition of mitophagy.

When the skin is exposed to UV, large amounts of ROS are produced in the mitochondria, which induces oxidative stress leading to mitochondrial damage. Treatment of UV-irradiated

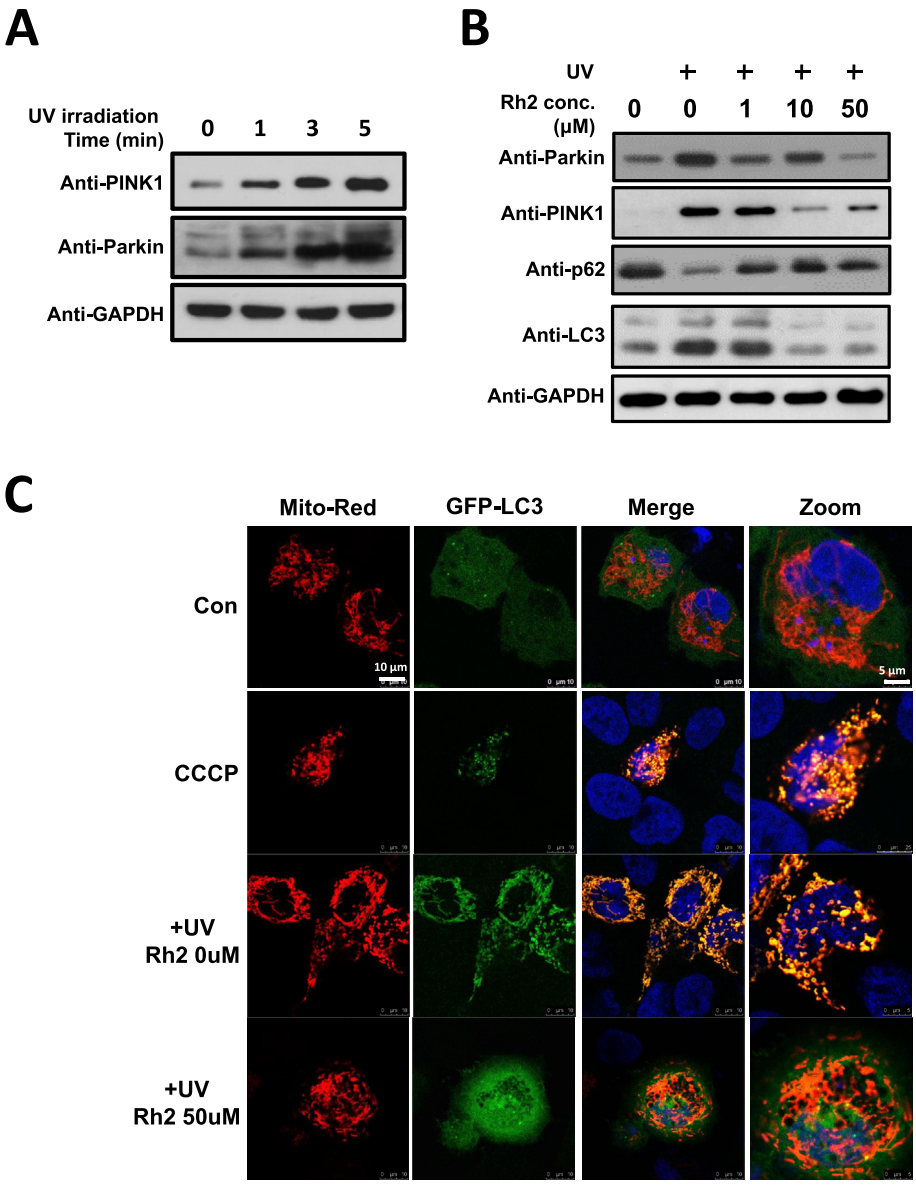

Fig. 6. Rh2 inhibits mitophagy in UV-irradiated NHDF cells. (A) Induction of mitophagy was measured by western blotting analysis with anti-PINK1 and *anti*-parkin antibodies after UV irradiation of 80 mJ/cm^3 for time-dependent manner. (B) The expression level of mitophagy related protein and autophagy protein in Rh2-treated cell line after UV exposure. (C) Image of MitoRed and GFP-LC3 staining from UV-exposed Rh2-treate samples. Scale bars, 10 μm or 5 μm.

NHDF cells with Rh2 extract increased ECM protein levels such as elastin. However, it reduced the levels of proteins involved in the inhibition of collagen synthesis, such as MMP-2 and MMP-3 (Fig. 3). Notably, Rh2 extract had no effects on the expression of the ECM protein, collagen. However, treatment with Rh2 extract ameliorated the UV-induced decrease in the level of elastin expression. Previously, we demonstrated that the ginsenoside, RG3, increased the protein expression levels of collagen, elastin, and COL1A1 that had been decreased by UV exposure [24]. Pathways that influence the effects of RG3 and Rh2 are presumably different. Because the only ECM-related protein recovered by Rh2 was elastin, the mechanism of action of Rh2 is potentially limited to the elastin synthesis pathway and may have no effect on the collagen synthesis pathway. Further research is needed to determine the interaction between Rh2 and elastin synthesis.

Rh2 also decreased oxidative stress by increasing the expression of antioxidant genes (Fig. 4), thereby reducing the generation of ROS by UV exposure. In addition, Rh2 inhibited UV-mediated mitophagy and restored the functions of mitochondria that had been reduced by UV damage (Figs. 5 and 6). Taken together, the findings in this study constitute clear in vitro evidence that Rh2 plays a positive role in UV-irradiated NHDF cells by protecting mitochondrial structure and function. Several studies have shown that Rh2 contributes to dermal fibroblast and keratinocyte regeneration; it also has antioxidant activities. This study demonstrated that Rh2 targets mitochondria and regulates the generation of ROS;

these findings will be useful in developing both methods for protection against UV and novel treatment strategies.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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