

Protective effects of rosmarinic acid against hydrogen peroxide-induced cellular senescence and the inflammatory response in normal human dermal fibroblasts

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Abstract. Hydrogen peroxide (H₂O₂) is a reactive oxygen species (ROS) that induces numerous cellular events, including cellular senescence and inflammatory responses. Therefore, the aim of this study was to investigate the protective effect of Rosmarinic acid (RA) in H₂O₂-induced oxidative stress in normal human dermal fibroblasts (NHDFs). Cytotoxicity assays were performed using a water-soluble tetrazolium salt, and senescence-associated β -galactosidase activity was determined to investigate the proportion of senescent cells. Antioxidant capacities were evaluated via H₂O₂-scavenging activity, reverse transcription-quantitative polymerase chain reaction, NRF2 luciferase reporter gene activity and intracellular ROS scavenging assays. Cytokine-coded gene expression analysis and nuclear factor- κ B luciferase activity were determined to verify the anti-inflammatory effect of RA. As a result, the present study demonstrated that rosmarinic acid inhibited H₂O₂-induced oxidative stress and inflammatory responses in normal human dermal fibroblasts. Initially, the doses of RA that exerted minimal cytotoxic effects in NHDFs were determined using a cytotoxicity assay. Subsequently, pretreatment with the appropriate doses of RA significantly reversed the H₂O₂-induced decrease in NHDF cell viability and decreased cellular senescence of NHDFs. In addition,

RA inhibited H₂O₂-induced ROS production in NHDFs, as determined by a ROS scavenging assay. The protective effects of RA were mediated by the inhibition of nuclear factor erythroid-derived 2-like 2, a transcription factor that functions as a key regulator of redox sensitivity. Furthermore, RA suppressed H₂O₂-induced inflammation in NHDFs and significantly rescued H₂O₂-induced downregulation of sirtuin 1. RA also inhibited nuclear factor (NF)- κ B transcriptional activity and the expression of NF- κ B target genes, including tumor necrosis factor- α and interleukin-6, in H₂O₂-exposed NHDFs. Taken together, these data indicate that RA inhibits H₂O₂-induced cellular damage in NHDFs.

Introduction

The term reactive oxygen species (ROS) refers to all chemically reactive molecules containing an oxygen atom, including oxygen radicals and non-radicals. The various types of ROS are classified as either endogenous mitochondrial factors or extracellular factors (1). Hydrogen peroxide (H₂O₂) is a reactive byproduct of the electron transport chain in mitochondria (1).

The skin is the largest organ of the body; it surrounds the body and protects it from the external environment. Extrinsic stimuli, including ultraviolet (UV) light, pollution and thermal stress, disrupt skin cell metabolism, thereby disrupting redox state equilibrium (2). Although cells employ efficient enzymatic and nonenzymatic antioxidant mechanisms, excessive ROS production can induce lipid peroxidation, DNA damage and protein denaturation (1). Proteins associated with conventional enzyme-mediated antioxidant mechanisms include superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT) (1). These enzymes are primarily activated in response to ROS. In H₂O₂-exposed normal human dermal fibroblasts (NHDFs), excessive ROS production disrupts antioxidant defense mechanisms and induces the inflammatory response (1,3).

In its inactive form in the cytoplasm, nuclear factor erythroid-derived 2-like 2 (NRF2) interacts with Kelch-like-ECH-associated protein 1 (4). A disruption in this interaction by factors, including ROS, activates the NRF2 signaling pathway (4). Activated NRF2 translocates to the

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nucleus where it binds to antioxidant response element (ARE), and initiates the transcription of genes associated with the response to oxidative stress, including heme oxygenase-1 (*HO-1*), *SOD* and *CAT* (5-7).

The mammalian Sir2 ortholog, sirtuin 1 (SIRT1), is a histone deacetylase that targets histones and nonhistone substrates, including nuclear factor (NF)- κ B and p53 (8,9). Previous studies have demonstrated that SIRT1 is associated with the regulation of numerous cellular processes, including inflammation, apoptosis and autophagy (10-12). Under conditions of adaptive stress, including hypoxia, infection and caloric restriction, SIRT1 inhibits the proinflammatory cytokine NF- κ B (13). NF- κ B serves a dominant role in inducing the inflammatory response (14,15). In response to excessive cellular stress, activated NF- κ B directly activates the expression of genes encoding key inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF)- α (16).

The use of the plant secondary metabolite rosmarinic acid (RA) has been studied in pharmaceutical and dietary supplements in Alzheimer's disease, atopic dermatitis and cardiovascular disease (17-21). Previous research has revealed that the effects of RA in these contexts are mediated by its antioxidant properties. One report demonstrated that the antioxidant activity of RA is able to inhibit UV-induced damage in the skin of mice (22). In addition, RA may protect human melanoma cells from H₂O₂-induced oxidative stress (23). Other reports have also demonstrated that RA protects human keratinocyte HaCaT cells from UVB-induced oxidative stress (24,25). However, the effects of RA on NHDFs, which are the key mediators of skin firmness and elasticity, remain unclear. The present study aimed to investigate the effects of RA on H₂O₂-exposed NHDFs.

Materials and methods

Cell culture and treatment. NHDFs (Lonza Group, Ltd., Basel, Switzerland) and the NF- κ B Luciferase Reporter NIH3T3 Stable Cell Line (Signosis, Inc., Santa Clara, CA, USA) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂. H₂O₂ (Sigma-Aldrich; Merck KGaA) was diluted in phosphate-buffered saline (PBS) to obtain a 1 M stock solution. For the H₂O₂ treatment experiments, the H₂O₂ stock solution was diluted in cell culture media at the indicated concentrations. RA (Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA).

Cell viability assay. Cytotoxicity was evaluated using a water-soluble tetrazolium salt (WST-1) assay (EZ-Cytox Cell Viability Assay kit; Itsbio, Seoul, Korea). NHDFs were seeded on 96-well culture plates at a density of 3x10³ cells and were incubated for 24 h, at 37°C. Subsequently, the cells were incubated with RA (0-50 μ M) for 12 h, after which the cytotoxicity of RA was assessed. To examine the effects of RA against H₂O₂-induced cytotoxicity, NHDFs were incubated with the indicated concentration of RA (10, 20 and

30 μ M) for 12 h prior to H₂O₂ treatment. After 12 h, 600 μ M of H₂O₂ was added and incubated at 37°C for 2 h. 1/10 volume of WST-1 solution was then added to each well, and the cells were incubated at 37°C for 1 h. Cell viability was evaluated by measuring absorbance at 450 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

H₂O₂ scavenging activity assay. The H₂O₂ scavenging activity of RA was evaluated as previously described (26). A solution containing H₂O₂ (40 mM in distilled water, pH 7.4) and varying concentrations of RA (0-1 mg/ml) was incubated at room temperature for 10 min. Scavenger activity was subsequently assessed by measuring the absorbance of the H₂O₂/RA solution at 230 nm using a UV spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A solution lacking H₂O₂ was used as a control, and L-ascorbic acid was used as the experimental control treatment. The experiments were conducted in triplicate at each concentration of RA. Scavenger activity was calculated using the following formula: H₂O₂ scavenging activity (%) = (1 - sample/control) x 100.

Intracellular ROS scavenging assay. Intracellular ROS production was evaluated using a 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich; Merck KGaA) staining assay. Briefly, cells were incubated with DCF-DA solution (100 μ M) at 37°C for 1 h, after RA and H₂O₂ treatment. ROS levels were then analyzed using flow cytometry. The proportion of fluorescence-positive cells was measured using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) with excitation and emission filters of 488 and 530 nm, respectively. *N*-acetyl-cysteine (NAC), ROS scavenger used as a positive control, was purchased from Sigma-Aldrich; Merck KGaA.

NF- κ B luciferase reporter assay. The NF- κ B luciferase reporter assay was conducted as previously described (27) with some modifications. Briefly, NF- κ B reporter NIH3T3 cells were seeded in 12-well cell culture plates at a density of 2x10⁵ cells/well. After 24 h incubation, cells were pretreated RA for 12 h at 10, 20 and 30 μ M, and were then treated with 600 μ M H₂O₂ for 2 h. At the end of the experiments, the cells were resuspended in Passive Lysis Buffer (Promega Corporation, Madison, WI, USA). Subsequently, luciferin (Sigma-Aldrich; Merck KGaA) was added to the cells, and luciferase activity was measured using a Veritas luminometer (Turner Designs, Sunnyvale, CA, USA). The luciferase activity was normalized to β -galactosidase (β -gal) activity, and relative activity is presented as the percentage activity of the control with standard deviation. The results represent the mean of three independent experiments.

Senescence-associated β -gal (SA- β -gal) activity. β -gal expression was used as a marker of senescence (28). β -gal expression levels were determined using an SA- β -gal staining kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. NHDFs were seeded at a density of 1x10⁶ cells/well in 60-mm cell culture plates and were incubated until cell confluence reached 90%. Later, cells were pretreated RA for 12 h at 10, 20 and 30 μ M, and were then treated with 600 μ M H₂O₂ for 2 h. After treatment, the cells

Table I. Primer sequences for quantitative polymerase chain reaction.

Gene name	Gene symbol	Forward (5'-3')	Reverse (5'-3')
Superoxide dismutase 1	<i>SOD1</i>	GGGAGATGGCCCAACTACTG	CCAGTTGACATGCAACCGTT
Catalase	<i>CAT</i>	ATGGTCCATGCTCTCAAACC	CAGGTCATCCAATAGGAAGG
Sirtuin 1	<i>SIRT1</i>	GCAGGTTGCGGGAATCCAA	GGCAAGATGCTGTTGCAAA
Tumor necrosis factor- α	<i>TNF-α</i>	CCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA
Interleukin-6	<i>IL-6</i>	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
Actin, beta	<i>β-actin</i>	GGATTCCTATGTGGGCGACGA	CGCTCGGTGAGGATCTTCATG

were washed in PBS and incubated in 0.5 ml fixative solution (4% formaldehyde, 0.5% glutaraldehyde in PBS buffer, pH 7.2) for 10 min at room temperature. The fixed cells were incubated in a staining solution mixture [staining solution (470 μ l), staining supplement (5 μ l) and 20 mg/ml X-Gal in dimethylformamide (25 μ l)] for 24 h at 37°C. Subsequently, 70% glycerol (1 ml) was added to the cells and blue SA- β -gal-positive cells were counted under a microscope (Olympus IX51; Olympus Corporation, Tokyo, Japan).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Before RNA extraction, cells were treated with RA (10, 20 and 30 μ M) for 12 h and H₂O₂ (600 μ M) for 2 h, in a consecutive manner. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA purity and concentration were evaluated using a MaestroNano[®] microspectrophotometer (Maestrogen, Las Vegas, NV, USA). Total RNA was reverse transcribed into cDNA using the oiligo d(T)₂₃ primer and ProtoScript First Strand cDNA Synthesis kit (New England BioLabs, Inc., Ipswich, MA, USA) following the manufacturer's instructions. *HO-1*, *SOD1*, *CAT*, *SIRT1*, *TNF- α* and *IL-6* mRNA expression levels were evaluated using 5x HOT FIREPOL[®] EvaGreen[®] qPCR mix (Solis BioDyne, Tartu, Estonia), a StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and gene-specific primers. Each thermo-cycling condition was same in denaturation (95°C) and extension (72°C), except the annealing temperature which differed when different primers were used. The C_q value associated with each gene was normalized to the expression of the β -actin housekeeping gene. The sequences of the qPCR primers are provided in Table I. The 2^{- $\Delta\Delta C_q$} method (29) was used to calculate the relative expression levels of each gene.

Analysis of luciferase reporter gene activity. Transcriptional activity of NRF2 was determined using an antioxidant ARE Reporter kit (cat. no. 60514; BPS Bioscience, San Diego, CA, USA). The luciferase activity was quantitatively assessed according to the manufacturer's instructions. Briefly, NHDFs were cotransfected with NRF2-ARE-luciferase reporter plasmid (BPS Bioscience) and a plasmid that constitutively expressed *Renilla* luciferase using Lipofectamine[™] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection was conducted according to the manufacturer's instructions with some modifications. The Lipofectamine reagent was

mixed with serum-free Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min, and then added to the plasmid with gently flicking for 20 min, at room temperature. The transfection mixture was added to the cells and incubated at 37°C. After 24 h of transfection, cells were pretreated for 12 h with the indicated doses of RA (10, 20 and 30 μ M), and were then exposed to 600 μ M H₂O₂ for 2 h. The cells were re-suspended in Passive lysis buffer (Promega Corporation) and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega Corporation). The luciferase activity of ARE was normalized to *Renilla* luciferase activity.

Statistical analysis. All results are presented as the mean \pm standard deviation of three independent experiments. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey post hoc test was conducted for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

RA reduces H₂O₂-induced cytotoxicity in NHDFs. The present study initially determined the dose range of RA that was not markedly cytotoxic to NHDFs. The cells were treated with increasing concentrations of RA (0-50 μ M) for 12 h. Concentrations of RA <50 μ M were not significantly cytotoxic; therefore, 10-30 μ M RA was used for subsequent experiments. To determine if RA affects the viability of NHDFs exposed to H₂O₂-induced cellular stress, NHDFs were sequentially treated with RA and H₂O₂, and cytotoxicity was evaluated using a WST-1 assay. As presented in Fig. 1, cell viability decreased to 76% of the control in cells treated with 600 μ M H₂O₂ for 12 h. Pretreatment with 10-30 μ M RA for 12 h significantly inhibited H₂O₂-induced cytotoxicity (Fig. 1B).

RA inhibits H₂O₂-mediated induction of SA- β -gal activity in NHDFs. H₂O₂-mediated ROS activates endogenous cellular responses to oxidative stress (30). However, when this defense system collapses, superoxide anion, hydroxyl radical and singlet oxygen production induces cellular senescence and apoptosis (31-33). In the present study, H₂O₂ induced cellular senescence in NHDFs, as demonstrated by an SA- β -gal assay (Fig. 2). Notably, RA markedly inhibited cellular senescence in a concentration-dependent manner in H₂O₂-exposed

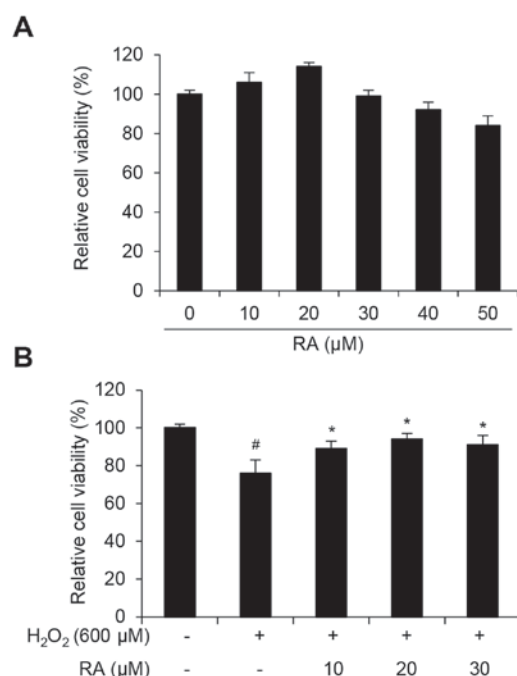


Figure 1. Protective effects of RA against H₂O₂ in NHDFs. (A) Evaluation of cytotoxicity in RA-treated NHDFs. Following 12 h of RA treatment, cell viability was evaluated using a water-soluble tetrazolium salt-based cytotoxicity assay. (B) RA mediated protective effects against H₂O₂ cytotoxicity in NHDFs. Cells were pretreated with varying doses of RA for 12 h prior to exposure to 600 μM H₂O₂. Data are presented as the mean ± standard deviation of relative cell viability from triplicate experiments. [#]P<0.05 compared with non-treated control cells; ^{*}P<0.05 compared with H₂O₂-treated cells. H₂O₂, hydrogen peroxide; NHDFs, normal human dermal fibroblasts; RA, rosmarinic acid.

NHDFs (Fig. 2), thus suggesting that RA protects NHDFs from H₂O₂-mediated induction of SA-β-gal activity.

RA inhibits H₂O₂-induced oxidative stress in NHDFs. Similar to previous studies, the present study investigated whether the protective effects of RA were associated with its oxidative properties (23-25). To evaluate the ROS scavenging activity of RA, H₂O₂ scavenging activity, DCF-DA fluorescence intensity, NRF2 activity and the expression of genes that regulate oxidative stress (*HO-1*, *SOD1* and *CAT*) were assessed. RA showed an increase in H₂O₂ scavenging activity and ascorbic acid was used as positive control (Fig. 3A). Although the free radical scavenging activity of RA was less than the positive control (L-ascorbic acid), the half maximal inhibitory concentration of RA was 0.56 mg/ml, and the radical scavenging activity of RA occurred in a dose-dependent manner. DCF fluorescence intensity, which is an indicator of intracellular ROS levels, was evaluated in RA-pretreated NHDFs with or without H₂O₂ exposure. DCF intensity increased >8-fold in H₂O₂-exposed cells compared with the nonexposed control group (Fig. 2B). However, this effect was strongly reduced in NHDFs pretreated with either RA or the positive control antioxidant NAC (Fig. 3B). NRF2 serves a key role in the regulation of antioxidant mechanisms by activating the transcription of genes encoding antioxidant enzymes, including *HO-1*, *SOD1* and *CAT*, via the ARE in the target gene promoter (5-7). Therefore, the present study analyzed the transcriptional activity of NRF2 using the ARE-luciferase assay.

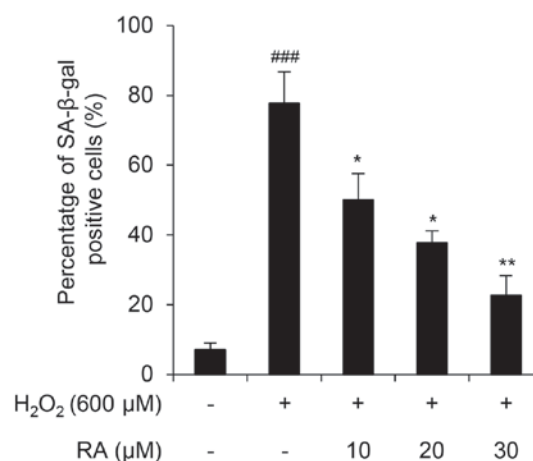


Figure 2. Protective effects of RA against H₂O₂-mediated SA-β-gal activation in NHDFs. Cells were pretreated with the indicated doses of RA for 12 h prior to exposure to 600 μM H₂O₂ for 48 h. Cellular senescence was evaluated using an SA-β-gal assay. Data are presented as the mean ± standard deviation from triplicate experiments. ^{###}P<0.001 compared with non-treated control cells; ^{*}P<0.05, ^{**}P<0.01 compared with H₂O₂-treated cells. H₂O₂, hydrogen peroxide; NHDFs, normal human dermal fibroblasts; RA, rosmarinic acid; SA-β-gal, senescence-associated β-galactosidase.

As presented in Fig. 3C, RA markedly enhanced luciferase activity in a concentration-dependent manner, thus suggesting that RA activates NRF2 activity in NHDFs. In addition, the expression levels of the NRF2 target genes, *HO-1*, *SOD1* and *CAT*, were detected. Notably, RA markedly upregulated the target genes against H₂O₂-induced oxidative stress (Fig. 3D). These results suggested that RA exerts a protective effect on H₂O₂-induced oxidative stress in NHDFs via NRF2-associated antioxidant mechanisms.

RA inhibits the H₂O₂-induced inflammatory response in NHDFs. The mammalian Sir2 ortholog, SIRT1, is a histone deacetylase that targets histones and nonhistone substrates, including NF-κB and p53 (8,9,34). Previous studies have demonstrated that SIRT1 is involved in the regulation of numerous cellular processes, including inflammation, apoptosis and autophagy, and that SIRT1 expression is down-regulated in response to oxidative stress (10-12,35). Therefore, the present study evaluated the expression levels of SIRT1 and various inflammatory cytokines, including NF-κB, TNF-α and IL-6 in NHDFs. *SIRT1* levels were decreased in NHDFs exposed to 600 μM H₂O₂, whereas RA inhibited this effect in a concentration-dependent manner (Fig. 4A). To determine the effects of H₂O₂ and RA on NF-κB expression, an NF-κB luciferase reporter stable NIH-3T3 cell line was used. As presented in Fig. 4B, relative luciferase activity in H₂O₂-exposed NHDFs increased 5.71±0.42-fold compared with the control group (P<0.05). Notably, pretreatment with 20 and 30 μM RA significantly inhibited this effect (P<0.05). To further evaluate the effects of H₂O₂ and RA on the activity of NF-κB, the expression levels of the NF-κB target genes, *TNF-α* and *IL-6*, were determined. H₂O₂ upregulated *TNF-α* and *IL-6* expression, whereas RA significantly inhibited this effect in a concentration-dependent manner (Fig. 4C). Taken together, these results indicated that RA exerts an anti-inflammatory effect on NHDFs under conditions of excessive oxidative stress.

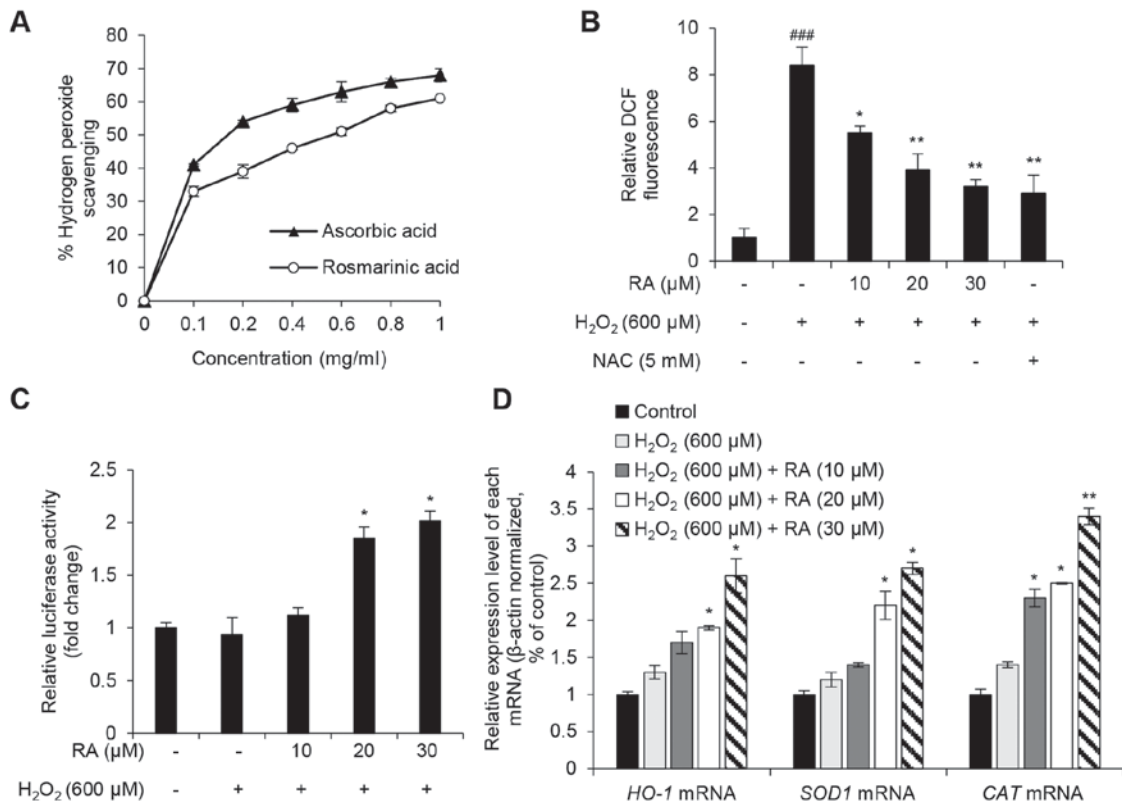


Figure 3. Antioxidative effects of RA in H₂O₂-exposed NHDFs. (A) H₂O₂ scavenging activity of ≥1 mg/ml RA. L-ascorbic acid was used as the positive control. (B) Intracellular ROS scavenging activity of RA in H₂O₂-exposed NHDFs. NAC was used as the positive control. (C) Effects of RA on nuclear factor erythroid-derived 2-like 2 transcriptional activity in H₂O₂-treated NHDFs. Transcriptional activity was evaluated using an antioxidant response element-luciferase reporter assay. (D) Effects of RA on the expression of antioxidant genes in H₂O₂-exposed NHDFs. *HO-1*, *SOD1* and *CAT* mRNA expression levels were evaluated using quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation from triplicate experiments. ###P<0.001 compared with non-treated control cells; *P<0.05, **P<0.01 compared with H₂O₂-treated cells. *CAT*, catalase; DCF, 2,2'-dichlorofluorescein; H₂O₂, hydrogen peroxide; *HO-1*, heme oxygenase-1; NAC, *N*-acetyl-cysteine; NHDFs, normal human dermal fibroblasts; RA, rosmarinic acid; *SOD1*, superoxide dismutase.

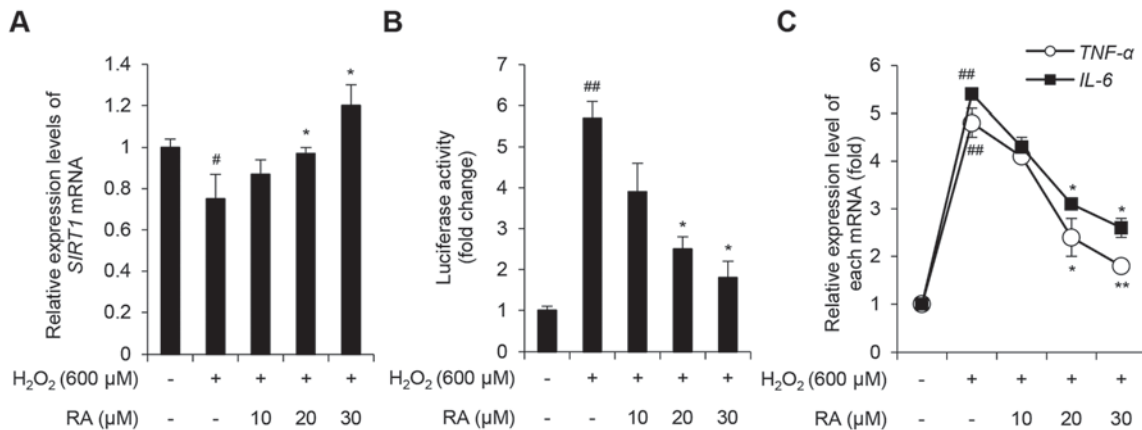


Figure 4. Effects of RA on the H₂O₂-induced inflammatory response in NHDFs. (A) Effects of RA on *SIRT1* expression in H₂O₂-exposed NHDFs. *SIRT1* expression was evaluated using qPCR. (B) Effects of RA on NF-κB activity in fibroblasts. NF-κB activity was evaluated using an NF-κB-luciferase reporter assay. (C) Effects of RA on the expression of genes associated with inflammation in H₂O₂-exposed NHDFs. *TNF-α* and *IL-6* expression levels were evaluated using qPCR. Data are presented as the mean ± standard deviation from triplicate experiments. #P<0.05, ##P<0.01 compared with non-treated control cells; *P<0.05, **P<0.01 compared with H₂O₂-treated cells. H₂O₂, hydrogen peroxide; *IL-6*, interleukin-6; NF-κB, nuclear factor-κB; NHDFs, normal human dermal fibroblasts; qPCR, quantitative polymerase chain reaction; RA, rosmarinic acid; *SIRT1*, sirtuin 1; *TNF-α*, tumor necrosis factor-α.

Discussion

Numerous studies have demonstrated that ROS exist in all living tissues, and are essential for various cellular functions, including epithelial cell proliferation and wound healing (36),

and dermal fibroblast migration (37). However, excess ROS can trigger deleterious effects, such as apoptosis and cellular senescence (3). There is a marked decreased in antioxidant levels in aged skin, and treating aged skin with antioxidants, such as resveratrol and vitamin E, can partially rejuvenate

aged skin (38). These findings suggested that ROS may be the primary cause for the appearance of aging skin. H₂O₂ is a type of ROS, which is generated in response to UV light and numerous pollutants (39). The histological alterations in aged skin are markedly visible in the dermis layer, where dermal fibroblasts are the primary cell type (40). In addition, a reduction in CAT activity and an increase in H₂O₂ levels have been observed in dermal fibroblasts from aged human skin compared with in dermal fibroblasts from younger human skin (41). In a previous study, treatment with exogenous CAT rescued the reduction in intracellular CAT protein activity and the increase in H₂O₂ levels in aged fibroblasts, and also reduced collagenase matrix metalloproteinase 1 expression (41). These data indicated that suppressing H₂O₂ generation in dermal fibroblasts may inhibit the skin aging process. Notably, in the present study, H₂O₂ exposure strongly enhanced cytotoxicity and SA- β -gal activity, which are similar to the effects observed in tissues from older individuals (42). However, RA pretreatment markedly protected NHDFs from H₂O₂-induced cytotoxicity and SA- β -gal activation. In addition, the H₂O₂ scavenging capacity of RA was investigated, as was the intracellular H₂O₂ scavenging capacity of RA in NHDFs. The results demonstrated that RA exhibited substantial H₂O₂ scavenging activity and inhibited H₂O₂-induced intracellular ROS production. Taken together, these data suggested that RA may be considered a potential ingredient in anti-aging skin products.

The results of the present study prompted the hypothesis that RA may regulate intracellular antioxidant activity in NHDFs. Although RA has exhibited antioxidant activity in keratinocytes (24,25), there are differences in the oxidative stress response between keratinocytes and dermal fibroblasts (43). To test the hypothesis, ARE luciferase assays and RT-qPCR were conducted to evaluate the expression of the antioxidant-induced NRF2 target genes *HO-1*, *SOD1* and *CAT*. RA rescued H₂O₂-mediated inhibition of NRF2 transcriptional activity and the consequential decrease in NRF2 target gene expression. Consistent with these findings, a previous study demonstrated that RA inhibits UVB-induced ROS production and the decrease in protein levels encoded by NRF2 target genes in HaCaT keratinocytes (24). In addition, a previous report demonstrated that the NRF2-inducer tanshinone I exerted a protective effect against UV radiation in human skin cells and reconstructed human skin (44). Furthermore, *NRF2* depletion induces damage to the extracellular matrix (45), a hallmark of skin aging (46). Taken together, these findings indicated that RA may inhibit H₂O₂ via a NRF2-associated antioxidant mechanism in NHDFs.

The present study also demonstrated that RA inhibited the H₂O₂-induced inflammatory response in NHDFs. Oxidative stress is associated with inflammation in skin tissue (47), and upregulation of the inflammatory response is associated with numerous skin diseases, including atopic dermatitis and psoriasis (48). SIRT1 serves critical roles in apoptosis, senescence, autophagy, gene silencing and inflammation (11,12,48,49). Under excessive ROS conditions, SIRT1 is inactivated and cannot suppress the activity of NF- κ B, which is a protein that serves a key role in inflammatory responses by activating the transcription of proinflammatory cytokines, such as TNF- α and IL-6 (16). In the present study, RA inhibited H₂O₂-mediated *SIRT1* downregulation in NHDFs, and NF- κ B activity was

decreased in RA-treated NHDFs. Furthermore, RA significantly downregulated the expression levels of TNF- α and IL-6 in a dose-dependent manner. NF- κ B activation is regarded as a potential biomarker of aging (14); an increase in NF- κ B activity is correlated with tissue aging and age-associated degenerative diseases (15).

In conclusion, the findings of the present study suggested that RA may inhibit senescence and inflammatory responses in NHDFs, which are effects associated with aging skin. To gain insight into the effects of RA on human skin, further studies should focus on the efficacy of RA in reversing the signs of aging skin and evaluate the precise molecular mechanisms by which RA mediates this effect *in vivo*.

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