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Limonene protects human skin keratinocytes against UVB-induced photodamage and photoaging by activating the Nrf2-dependent antioxidant defense system

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Abstract

Long term exposure to solar ultraviolet B (UVB) radiation is one of the primary factors of premature skin aging and is referred to as photoaging. Also, mammalian skin exposed to UVB triggers an increase in production of α-melanocyte-stimulating hormone (α-MSH), which is critically involved in the pathogenesis of hyperpigmentary skin diseases. This study investigated the protective effect of limonene on UVB-induced photodamage and photoaging in immortalized human skin keratinocytes (HaCaT) in vitro. Initially, we determined cell viability and levels of reactive oxygen species (ROS) in UVB-irradiated HaCaT cells. Pretreatment with limonene increased cell viability followed by inhibition of intracellular ROS generation in UVB-irradiated HaCaT cells. Interestingly, the antioxidative activity of limonene was directly correlated with an increase in expression of endogenous antioxidants, including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO-1), and γ-glutamylcysteine synthetase (γ-GCLC), which was associated with enhanced nuclear translocation and activation of NF-E2-related factor-2 (Nrf2). Indeed, Nrf2 knockdown reduced limonene's protective effects. Additionally, we observed that limonene treatment inhibited UVB-induced α-MSH secretion followed by inhibition of proopiomelanocortin (POMC) via suppression of p53 transcriptional activation. Moreover, limonene prevented UVB-mediated depletion of tight junction regulatory proteins, including occludin and zonula occludens-1. On the other hand, limonene treatment significantly decreased matrix metalloproteinase-2 levels in UVB-irradiated HaCaT cells. Based on these results, limonene may have a dermato-protective effect in skin cells by activating the Nrf2-dependent cellular antioxidant defense system.

KEYWORDS
antioxidant, HaCaT, limonene, Nrf2 pathway, photoaging, photodamage, UVB

1 | INTRODUCTION

Exposure to solar ultraviolet light, especially ultraviolet B (UVB) wavelengths (280–320 nm), plays a crucial role in the development of photoaging, photodermatosis, melanoma, and nonmelanoma skin cancers.[1–3] In skin cells, UVB radiation induces reactive oxygen species (ROS) generation, which can lead to DNA damage caused by oxidative stress.[4] Even though inhibiting ROS-induced oxidative stress may help prevent photoaging, several substances or extracts have demonstrated the ability to protect against ROS-induced photoaging.

Although, skin is equipped with a cellular antioxidant defense system that neutralizes UVB-induced ROS generation and protect skin

from harmful effects of UVB.[5] An increase in the antioxidant defense system, whether non-enzymatic or enzymatic prevents the deleterious effects of UVB-induced ROS on normal skin, such as photocarcinogenesis and photoaging.[6] The cellular responses to oxidative stress involves signaling proteins that act through the antioxidant response element (ARE) and the transcription factor NF-E2-related factor-2 (Nrf2).[7] ARE mainly regulates the expression of phase II detoxifying enzyme genes. Under normal conditions, Nrf2 is sequestrated with its inhibitor Keap-1 in the cytoplasm, upon activation, Nrf2 dissociates from the Keap1-Nrf2 complex and translocates into the nucleus where it binds with the ARE promoter region to enhance the expression of phase II genes such as heme oxygenase (HO-1), NAD(P)H:quinine oxidoreductases (NQO1), glutathione reductase (GR), glutathione-S-transferase (GST), and superoxide dismutase (SOD).[7] These antioxidant genes play vital roles in protecting cells from free radical stress imposed by ROS.[8]

UV-induced DNA damage and repair are largely controlled by the tumor suppressor protein p53. Exposure of dermal keratinocytes to UV radiation results in p53 phosphorylation at serine 389 residue. As a transcription factor, the activated p53 binds directly to the proopiomelanocortin (POMC) gene promoter in such keratinocytes and induced POMC transcription and α-melanocyte-stimulating hormone (α-MSH) production, *which* is a key factor in the biosynthesis of melanin in melanocytes.[9,10] A previous study by Kong et al.[11] demonstrated that keratinocytes exposed to physiological doses of UVB markedly increased p53 activation through the ROS-p38 MAPK cascade.

Studies have revealed that during the photoaging, tight junction molecules (TJs), such as claudins, occludins, and junctional adhesion molecules (JAM-A and JAM-B), and plaque proteins such as ZO-1, ZO-2, ZO-3, MUPP-1, and symplekin were modulated by UV radiation.[12] A previous study by Yuki et al.[13] have demonstrated that UVB irradiation remarkably reduced occludin expression and function in skin keratinocytes. Furthermore, the basement membrane proteins, including matrix metalloproteinases (MMPs) and urinary plasminogen activator (uPAs) are increased in UV-irradiated skin. A proteomic study demonstrated that the exposure of human epithelial keratinocytes to UVB causes an increase in ROS generation, which may lead to oxidative damage to proteins involved in skin barrier function.[14]

Numerous studies have found that terpenoids are potent natural antioxidants, which provide protection under oxidative conditions in various organs including, liver, skin, brain, renal, and cardiovascular system, as well as in the aging process.[15] Limonene, a naturally occurring cyclic monoterpene, can be obtained from variety of fruits and vegetables, such as citrus and conifer plants. Indeed, limonene is one of the major compounds in citrus essential oils that are widely used as flavoring agents for foods, cosmetics, medicines, personal care products, and cleaning products is consistently increased due to its high-quality fragrance property.[16] Several studies have demonstrated that limonene possess various bioactivities including antioxidant, antidiabetic, anticancer, anti-inflammatory, cardioprotective, gastroprotective, hepatoprotective, immune-modulatory, anti-fibrotic, anti-genotoxic effects.[16] Recently, we have reported that limonene isolated from leaf and rhizome essential oils of *Alpinia nantoensis* inhibits forskolin-induced melanin production in murine melanoma cells.[17] However, the effect of limonene on UVB-induced photodamage and photoaging has yet to be studied. Therefore, the aim of the present study was to investigate the effect of limonene on UVB-induced oxidative stress and premature aging in human skin keratinocytes (HaCaT) in vitro.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Limonene was isolated from the leaf and rhizome essential oils of *Alpinia nantoensis*.[17] The purity was above 99%, as confirmed by HPLC and ^{1}H-NMR analysis. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies, Grand Island, NY. 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA), 4′-6-diamidino-2-phenylindole (DAPI), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO). Specific pharmacological inhibitors for JAN/SAPK and AKT/PI3K were obtained from Calbiochem (La Jolla, CA) and Santa Cruz Biotechnology, Inc (Dallas, TX), respectively. Antibodies against phos-AKT, AKT, phos-JNK/SAPK, phos-p53, histone, claudin-1, ZO-1 and γ-GCLC were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against MMP-2, β-actin, and GAPDH were bought from Santa Cruz Biotechnology, Inc. Antibodies against HO-1, NQO-1, and Nrf2 were procured from Abcam (Cambridge, UK). All other chemicals were reagent grade or HPLC grade and supplied by either Merck (Darmstadt, Germany) or Sigma-Aldrich.

2.2 | Cell culture and cell viability assay

HaCaT cells (an immortalized human keratinocyte cell line) was kindly gifted from Dr. P.S. Lai (National Chung Hsing University, Taichung, Taiwan). Cells were cultured in DMEM supplemented with 10% FBS, glucose, penicillin, and streptomycin and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Cell viability was assessed by MTT colorimetric assay. Briefly, HaCaT cells were seeded into a 24-well culture plate with a density of 5×10^4 cells/well. After 24 h incubation, cells were treated with increasing concentrations of limonene (12.5–200 μM) for 24 h. On the other hand, HaCaT cells were seeded onto 6-well plates with a density of 1×10^5 cells/well. After 24 h incubation, culture medium was replaced with phosphate buffer saline (PBS) and cells were exposed to different doses of Stratalinker 2400 equipped with 312-nm UVB bulbs (Stratagene, La Jolla, CA). Following UVB exposure, fresh DMEM was substituted for PBS, and incubation was continued for 24 h. For the comparative control, HaCaT cells were incubated with 500 μM H_2O_2 for 24 h. Control cells were incubated with 0.1% DMSO for 24 h. Cell culture supernatant was removed, and 1 mg/ml of MTT in 0.4 ml fresh culture medium

was added. The MTT formazan crystals were dissolved in 0.4 ml of DMSO, and the samples were measured at 570 nm (A_{570}) using an ELISA microplate reader, Bio-Tek Instruments (Winooski, VT). The percentage of cell viability was calculated as (A_{570} of treated cells/A_{570} of untreated cells) × 100.

2.3 | Determination of intracellular ROS generation

Intracellular ROS accumulation in HaCaT cells was determined using the fluorescent marker $DCFH_2$-DA following a procedure described earlier with minor modifications.[18] Briefly, HaCaT cells at a density of 2×10^5 cells/dish were seeded in 6 cm dish and preincubated with limonene (25–100 μM) or ascorbic acid (200 μM) for 2 h, then exposed to 50 mJ/cm^2 UVB, and further incubated for 1 h. At the end of the incubation, the culture supernatant was removed, and the cells were washed twice with phosphate buffered saline. $DCFH_2$-DA (10 μM) was mixed with 2 ml DMEM and added to the culture plate. After incubation for 30 min, relative fluorescence intensity was measured using a fluorescence spectrophotometer (Hidex Oy, Turku, Finland) at 485/535 nm ($A_{485/535}$). The percentage of ROS generation (%) was calculated as ($A_{485/535}$ of UVB + sample-treated cells/$A_{485/535}$ of control cells) × 100.

2.4 | Quantification of intercellular α-MSH

The amount of α-MSH in culture media was measured using a commercially available human α-MSH ELISA kit (Elabscience Biotechnology Inc, Hubei, China). HaCaT cells at a density of 2×10^5 cells/dish were seeded in 6 cm dishes and preincubated with limonene (25–100 μM) or gallic acid (100 μM) for 2 h, and then exposed to 50 mJ/cm^2 UVB and further incubated for 24 h. The culture media were subjected to immunoassay following supplier instruction. The immunoreactivity was measured at 450 nm using a micro-plate reader. In addition, the α-MSH standard curve was generated using known concentrations of α-MSH supplied by the ELISA kit. The amount of secreted α-MSH in culture media was calculated using α-MSH standard curve.

2.5 | Protein extraction and western blot analysis

HaCaT cells (1×10^6 cells/dish) were seeded in 10-cm dishes and preincubated with 0.1% dimethyl sulfoxide (control) or various concentrations of limonene (25–100 μM) or resveratrol (40 μM) or specific mitogen-activated protein kinases (MAPKs) inhibitors (30 μM) for 2 h, then exposed to UVB (50 mJ/cm^2). The UVB-challenged cells were further incubated for 30 min to 24 h. Cells were lysed by either mammalian protein extraction reagent or nuclear and cytoplasmic extraction reagent kit (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amount of protein samples (60 μg) were separated by 8%–12% SDS-PAGE and separated proteins were transferred onto polyvinylidene fluoride membrane overnight at 30 V for 10 h. The transferred protein membranes were blocked with 5% non-fat skim milk for 30 min, followed by incubation with specific primary antibodies overnight, and either horseradish peroxidase-conjugated anti-rabbit or anti-mouse or anti-goat antibodies for 2 h. Immunoblots were developed with enhanced chemiluminescence (ECL) reagents (Advansta Inc., San Jose, CA), images were captured by ChemiDoc XRS + docking system and the protein bands were quantified by Imagelab software (Bio-RadLaboratories, Hercules, CA).

2.6 | RNA extraction and q-PCR analyses

Total RNA was extracted by a total RNA purification kit (GeneMark, New Taipei City, Taiwan). The RNA concentration was quantified using a NanoVue Plus spectrophotometer (GE Health Care Life Sciences, Chicago, IL). Quantitative PCR (qPCR) was performed on a real-time PCR detection system and software (Applied Biosystems, Foster City, CA). First-strand complementary DNA (cDNA) was generated by SuperScript IV reverse transcriptase kit (Invitrogen). Quantification of mRNA expression for genes of interest was performed by qPCR reactions with an equal volume of cDNA, forward and reverse primers (10 μM), and Power SYBR Green Master Mix (Applied Biosystems). The primer sequences of each gene for qPCR were as follows. HO-1: forward primer (F), 5′-TCAACGGCACAGTCAAGG-3′; reverse primer (R), 5′-ACTCCACGACATACTCAGC-3′′ POMC: forward primer (F), 5′-AAGCGCTACGGCGGTTTC-3′; reverse primer (R), 5′-TCTTGTAG GCTTCTTGATGATG-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer (F), 5′-GATCATCAGCAATGCCTCCT-3′; reverse (R), 5′-TTCCTCTTGTGCTCTTGCTG-3′. The copy number of each transcript was calculated as the relative copy number normalized by the GAPDH copy number.

2.7 | Immunofluorescence microscopy analysis

HaCaT cells at a density of 2×10^4 cells/well were cultured in an 8-well glass Nunc Lab-Tek® chamber (ThermoFisher Scientific, Waltham, MA) and pretreated with 0.1% dimethyl sulfoxide (control) or limonene (100 μM) or resveratrol (40 μM) for 2 h, then exposed to UVB (50 mJ/cm^2). The UVB-challenged cells were further incubated for 1 h. After treatment, culture media was removed, and the cells were fixed in 4% paraformaldehyde for 15 min; cells were permeabilized with 0.1% Triton X-100 for 10 min, washed and blocked with 10% FBS in PBS, and then incubated for 2 h with either anti-Nrf2 or anti-phospho-p53 antibodies in 1.5% FBS. The cells were then incubated with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody for another 1 h in 6% bovine serum albumin (BSA). Next, the cells were stained with 1 μg/ml DAPI for 5 min, washed with PBS, and visualized using a fluorescence microscope (Motic Electric Group, Fujian, China) at 40 × magnification.

2.8 | ARE promoter activity

To examine the ARE promoter activity, we used a luciferase reporter assay system (Promega, Madison, WI). Briefly, HaCaT cells were seeded in a 24-well plate with a density of 5×10^4 cells/well, which had reached 70%–80% confluence were incubated for 5 h with serum-free DMEM did not contain antibiotics. Then the cells were transfected with either an ARE or β-galactosidase plasmids by pcDNA vector using Lipofectamine 2000 (Invitrogen). Following transfection with the vector or plasmid, cells were treated with or without various concentrations of limonene (12.5–100 μM) or resveratrol (40 μM) or JNK/SAPK or AKT inhibitors (30 μM) for 2 h, and then exposed to UVB (50 mJ/cm^2) for 1 h. Cells were lysed, and their luciferase activity was measured using a luminometer (Hidex Oy, Turku, Finland). Relative luciferase activity was normalized by β-galactosidase activity in the cell lysates.

2.9 | Nrf2 knock-down by siRNA

siRNA for Nrf2 was transfected with Lipofectamine RNAiMax (Invitrogen, Waltham, MA) according to the manufacturer's instructions. For transfection, HaCaT cells were plated in 6-well plates to give 40%–60% confluence at the time of transfection. The next day, the culture medium was replaced with 0.5 ml of Opti-MEM (Invitrogen, Waltham, MA), and the cells were transfected by using RNAiMAX transfection reagent. In a separate tube, 100 pM of siRNA was added to 0.5 ml of an Opti-MEM and RNAiMAX reagent mixture. The resulting siRNA/RNAiMAX mixture was incubated for an additional 25 min at room temperature to allow complex formation. Subsequently, the solution was added to the cells in the 6-well plates, giving a final transfection volume of 1 ml. After 6 h incubation, the transfection medium was replaced with 2 ml of standard growth medium and the cells were cultured at 37°C. Cells were preincubated with limonene (100 μM) for 2 h and then exposed to UVB (50 mJ/cm^2). The UVB-challenged cells were further incubated for 1–24 h. Cells were lysed and subjected to ROS assay.

2.10 | Trans-epithelial electrical resistance measurement

The trans-epithelial electrical resistance (TEER) was determined to assess the integrity of the monolayer using an Evometer (Evom,[2] World Precision Instruments; Hertfordshire, UK) fitted with "chopstick" electrodes. HaCaT cells were grown on 24-well Millicell hanging cell culture insert with a pore size of 8.0 μm. A confluent monolayer of cells with an epithelial resistance of above 1000 Ω•cm^2 was used for subsequent experiments. In all groups except control and UVB alone, cells were pretreated with different doses of limonene for 2 h, after which media was decanted from culture plates and inserts, and cells were washed with PBS. PBS was added to the basolateral and apical compartments with a volume of 1 and 0.2 ml, respectively. After UVB exposure (50 mJ/cm^2), PBS was replaced with fresh culture media, and the plates were incubated for 48 h. In this incubation period, epithelial resistance was measured at various time points (6, 12, 24, and 48 h). An insert without cells was used as a blank and its mean resistance was subtracted from all samples. Measurements were taken three times per well, and the average resistance value was calculated.

2.11 | Statistical analysis

Data are expressed as mean ± SD of three independent experiments. All data were analyzed using the statistical software Graphpad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). Statistical analysis was performed using one-way ANOVA followed by Dunnett's test for multiple comparisons.

3 | RESULTS

3.1 | Cytotoxicity of limonene and UVB on HaCaT cells

A cytotoxicity study of limonene was performed before evaluating its dermato-protective properties. HaCaT cells were incubated with increasing doses of limonene (12.5, 25, 50, 100, and 200 μM) for 24 h, and cell viability was determined by MTT colorimetric assay. It was shown that limonene does not affect cellular viability up to a concentration of 100 μM for 24 h. While cell viability was significantly reduced with a concentration greater than 100 μM (Figure 1A). Next, we selected non-cytotoxic concentrations of limonene (below 100 μM) for further experiments. In addition, HaCaT cells exposed to UVB (25, 50, and 100 mJ/cm^2) or H_2O_2 (500 μM) for 24 h exhibited a dose-dependent reduction in cell viability. Particularly, treatment with 50 and 100 mJ/cm^2 UVB reduced cell number to 39.4% and 9.13%, respectively, compared to control cells (Figure 1B). Next, we examined the protective effects of limonene on UVB (50 mJ/cm^2)-induced reduction in cell viability. Pretreatment with limonene (25–100 μM) significantly reversed the effects of UVB-induced cell death in HaCaT cells. Indeed, compared with UVB-only treated cells, the cell viability was markedly increased to two-fold by pretreatment with limonene for 2 h (Figure 1C).

3.2 | Limonene prevents UVB-induced oxidative stress in HaCaT cells through the induction of endogenous antioxidants

HaCaT cells were exposed to UVB (50 mJ/cm^2), and the intracellular oxidation state was determined by fluorescence spectrophotometer with $DCFH_2$-DA as the fluorescent probe. The intracellular ROS content was significantly elevated in UVB-irradiated cells (6.19-fold), whereas pretreatment with limonene significantly as well as dose-

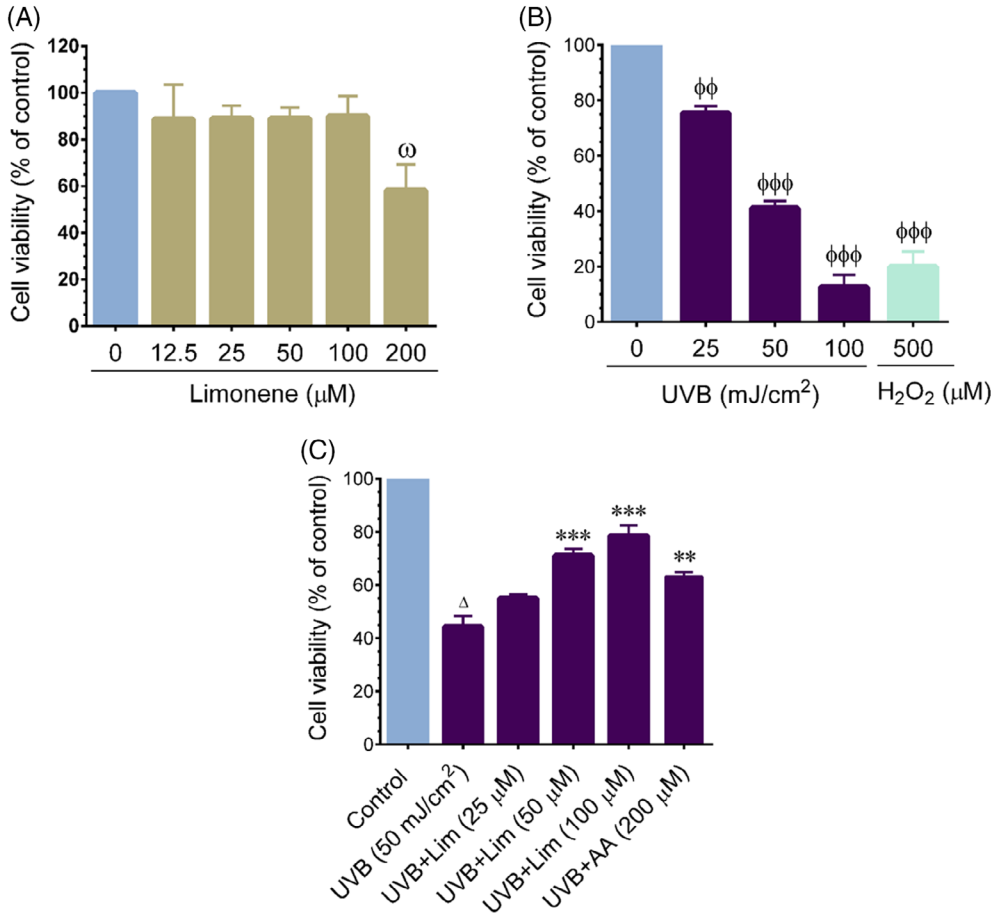

FIGURE 1 Cytotoxic effect of limonene and UVB on HaCaT cells. (A) HaCaT cells were incubated with increasing concentrations of limonene (12.5–200 μM) for 24 h. (B) HaCaT cells exposed to various doses of UVB (25–100 mJ/cm^2). (C) HaCaT cells were pretreated with indicated concentrations of limonene and ascorbic acid (AA) for 2 h and then exposed to UVB (50 mJ/cm^2) for 24 h. The cell viability was measured by MTT assay. The percentage of cell viability was compared with the control (0.01% DMSO) group. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\omega}p < .05$ compared to control versus limonene treatment group. $^{\phi\phi}p < .01$, and $^{\phi\phi\phi}p < .001$ compared with control versus UVB/H_2O_2 treatment groups. $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared with UVB alone treatment group versus UVB + sample treatment groups. HaCaT, human skin keratinocytes; UVB, ultraviolet B

dependently decreased the ROS content in UVB-irradiated HaCaT cells (Figure 2A). Next, we examined whether limonene possesses direct free radical scavenging properties; to that end a cell-free DPPH free-radical scavenging assay was performed with increasing concentrations of limonene and ascorbic acid (Vitamin C). Limonene showed a moderate level of free radical scavenging effects with an IC_{50} value of 392.81 μM. In contrast, ascorbic acid, a known antioxidant showed intense free radical scavenging activity with an IC_{50} value of 44.01 μM (Figure 2B). Thus, we speculate that limonene could activate the endogenous antioxidant system, which may further explain its strong antioxidant efficacy against UVB-induced oxidative stress. Here we measured the effects of limonene on endogenous antioxidants, including HO-1, NQO-1, and γ-GCLC after exposed to UVB-irradiated with or without limonene. As shown in Figure 2C, a significant increase in HO-1 mRNA level was observed in UVB-irradiated cells, while a remarkable increase of HO-1 mRNA was observed in limonene pretreated cells. This effect was further confirmed by western blotting, UVB treatment increased HO-1 and decreased NQO-1 protein levels, but these changes were not statistically significant. While UVB treatment significantly reduced γ-GCLC level in HaCaT cells. Indeed, pretreatment with limonene significantly increased HO-1, NQO-1, and γ-GCLC protein levels in UVB-irradiated HaCaT cells (Figure 2D).

3.3 | Limonene up-regulates antioxidative activity via Nrf2 pathway

Nrf2, a redox-sensitive essential leucine zipper transcription factor that responds to oxidative stress by up-regulating several ARE-dependent antioxidant genes, including HO-1, NQO-1, γ-GCLC, GCS, GPx, and so forth.[19] To determine whether limonene-mediated induction of antioxidative activity was associated with Nrf2 activity, ARE promoter activity was determined by luciferase reporter assay. As

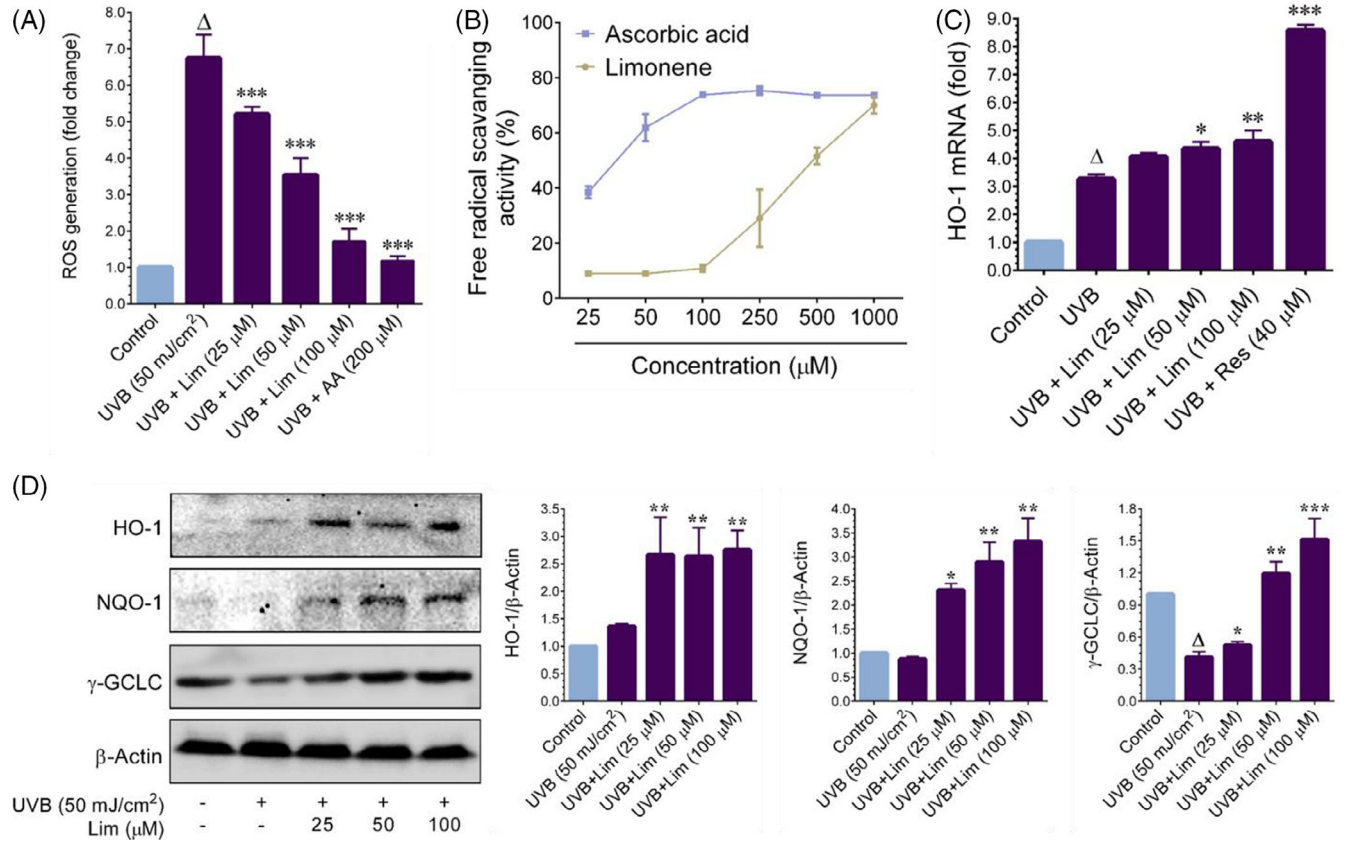

FIGURE 2 Limonene prevents UVB-induced oxidative stress in HaCaT cells through the induction of endogenous antioxidants. (A) Limonene inhibits UVB-induced intracellular ROS generation in HaCaT cells. (B) The free radical scavenging activity of limonene was determined by DPPH assay. Ascorbic acid (AA) was used as positive control. (C) To quantify the mRNA expression level of HO-1, HaCaT cells were preincubated with limonene (25–100 μM) or resveratrol (40 μM) 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 6 h. Total RNA was extracted and subjected to q-PCR analysis. Relative HO-1 mRNA level was normalized with GAPDH mRNA. (D) To determine the protein expression levels of HO-1, NQO-1, and γ-GCLC, HaCaT cells were preincubated with limonene (25–100 μM) for 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 24 h. Total cell lysates were prepared and subjected to western blot analysis. Histogram shows the relative protein expression levels of HO-1, NQO-1, and γ-GCLC, which are normalized with an internal control β-actin. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared with UVB alone versus UVB + sample treatment groups. HaCaT, human skin keratinocytes; q-PCR, quantitative-PCR; ROS, reactive oxygen species; UVB, ultraviolet B

shown in Figure 3A, luciferase activity in HaCaT cells transfected with the ARE reporter construct was increased by UVB however, the increased activity was not statistically significant. Whereas more than a five-fold increase in luciferase activity was observed in limonene pretreatment cells, which is highly comparable with resveratrol's activity. Nuclear export of cytoplasmic Nrf2 is another hallmark involved in Nrf2 transcriptional activity; immunofluorescence analysis enumerated UVB exposure moderately increased Nrf2 nuclear translocation, while a significant Nrf2 accumulation in the nucleus was observed in cells that were pretreated with limonene (Figure 3B). To further demonstrate the involvement of Nrf2 in limonene-mediated antioxidant effects, we employed the Nrf2 gene knock-down model using Nrf2 specific siRNA. As shown in Figure 3C, a 2.6-fold increase in intracellular ROS was observed in response to UVB in scrambled siRNA (control siRNA)-transfected cells. In contrast, pretreatment with limonene significantly blocked this increase (1.2-fold) and maintained the ROS levels to the basal level. Although, exposure of HaCaT cells to UVB displayed a 3.1-fold increase of intracellular ROS in siNrf2-transfected cells. Interestingly, partial inhibition of ROS production was observed in limonene pretreated cells under similar conditions (2.4-fold), this partial inhibition may be achieved through their direct free radical scavenging ability. These data strongly support our hypothesis that limonene eliminates UVB-mediated excessive ROS production through the induction of the Nrf2-dependent antioxidant system.

3.4 | Limonene promotes Nrf2 activity via activation of JNK/SAPK and AKT kinases

Activation of MAPKs family proteins, including p38 MAPK, ERK1/2, and JNK/SAPK, and AKT/PI3K trigger Nrf2 transcriptional activity followed by nuclear export.[20] Therefore, we examined whether MAPKs

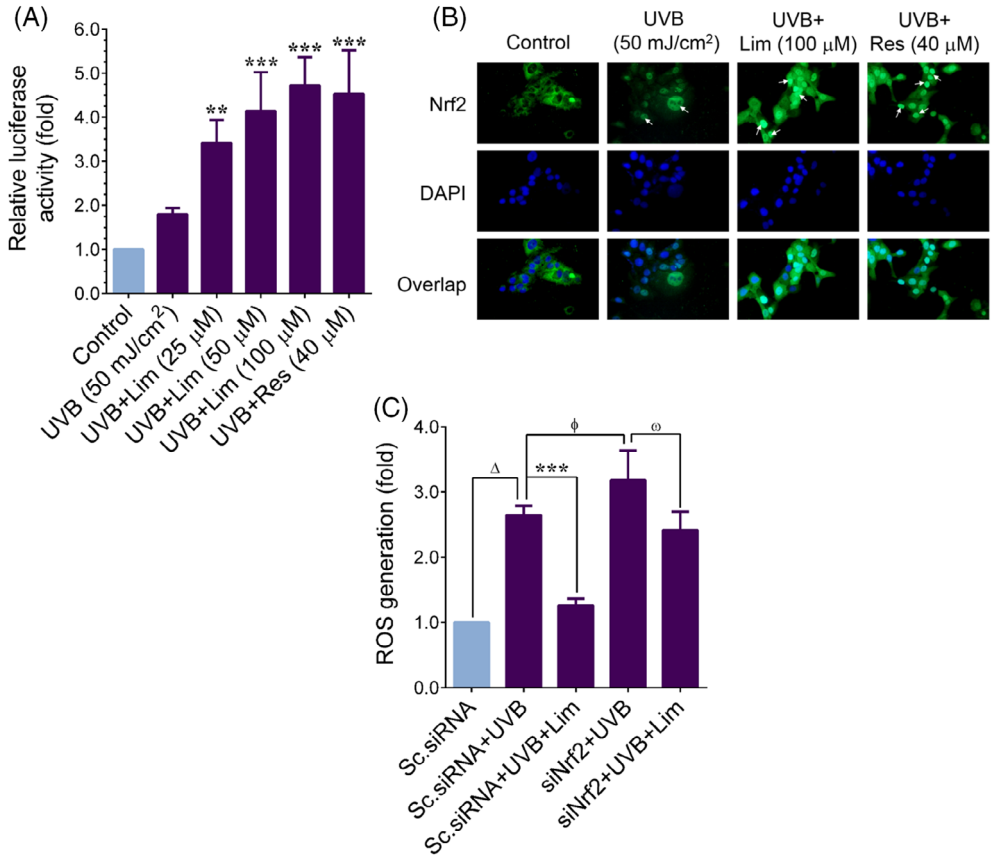

FIGURE 3 Limonene up-regulates antioxidants via Nrf2 pathway. (A) To determine the Nrf2 transcriptional activity, HaCaT cells were transiently transfected with ARE promoter construct using lipofectamine and preincubated with limonene (25–100 μM) or resveratrol (40 μM) for 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 6 h. Cell lysates were mixed with luciferase reagents and quantified using an illuminometer. Relative ARE promoter activity was calculated by dividing the relative luciferace unit (RLU) of treated cells by RLU of untreated cells (control). (B) To determine the nuclear localization of Nrf2, HaCaT cells were preincubated with limonene (25–100 μM) or resveratrol (40 μM) for 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 2 h. The protein expression and localization of Nrf2 was measured by immunofluorescence using Nrf2 specific primary antibody with FITC-conjugated secondary antibody (green). DAPI (1 μM) was used to stain the nucleus. (C) HaCaT cells were transfected with specific siRNA against Nrf2 or control siRNA. After transfection for 24 h, cells were preincubated with limonene (100 μM) for 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 1 h. Intracellular ROS was measured by $DCFH_2$-DA assay. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{**}p < .01$, $^{***}p < .001$ compared with UVB alone versus UVB + sample treatment groups. $^{\omega}p < .05$ compared to control siRNA + UVB versus siNrf2 + UVB. $^{\phi}p < .05$ compared with siNrf2 + UVB versus siNrf2 + UVB + limonene treatment group. HaCaT, human skin keratinocytes; Nrf2, NF-E2-related factor-2; ROS, reactive oxygen species; UVB, ultraviolet B

and AKT/PI3K pathways were involved in limonene-mediated Nrf2 activity in HaCaT cells. As shown in Figure 4A, a significant increase in JNK/SAPK and PI3K/AKT phosphorylation were observed in UVB-treated cells in the presence of limonene, whereas there was no significant increase in either p38 MAPK or ERK1/2 levels in limonene-treated cells (data not shown). To further demonstrate the requirement of JNK/SAPK and PI3K/AKT activation for limonene-mediated Nrf2 activity, ARE promoter activity was examined under pharmacological inhibition of JNK/SAPK and PI3K/AKT by SP600125 and LY294002, respectively. As evidenced from the data in Figure 4B, co-treatment with SP600125 and LY294002 significantly reduced the limonene-mediated Nrf2 promoter activity in a similar condition. Furthermore, western blot analysis confirmed that limonene treatment significantly increased nuclear accumulation of Nrf2, as indicated by increased Nrf2 protein level in the nuclear fraction, while this increase was significantly inhibited by both AKT and JNK/SAPK inhibitors treated cells (Figure 4C). Hence, these data indicate that limonene promotes Nrf2 transcriptional activity through activation of JNK/SAPK and PI3K/AKT pathways.

3.5 | Limonene inhibits UVB-induced α-MSH secretion in HaCaT cells

UVB radiation induces keratinocytes to express POMC via ROS/p53 axis, leads to the production of α-MSH, which interact with melanocortin-1 receptor (MC1R) in melanocytes to promote melanogenesis via activation of cAMP/PKA-dependent CREB/MITF

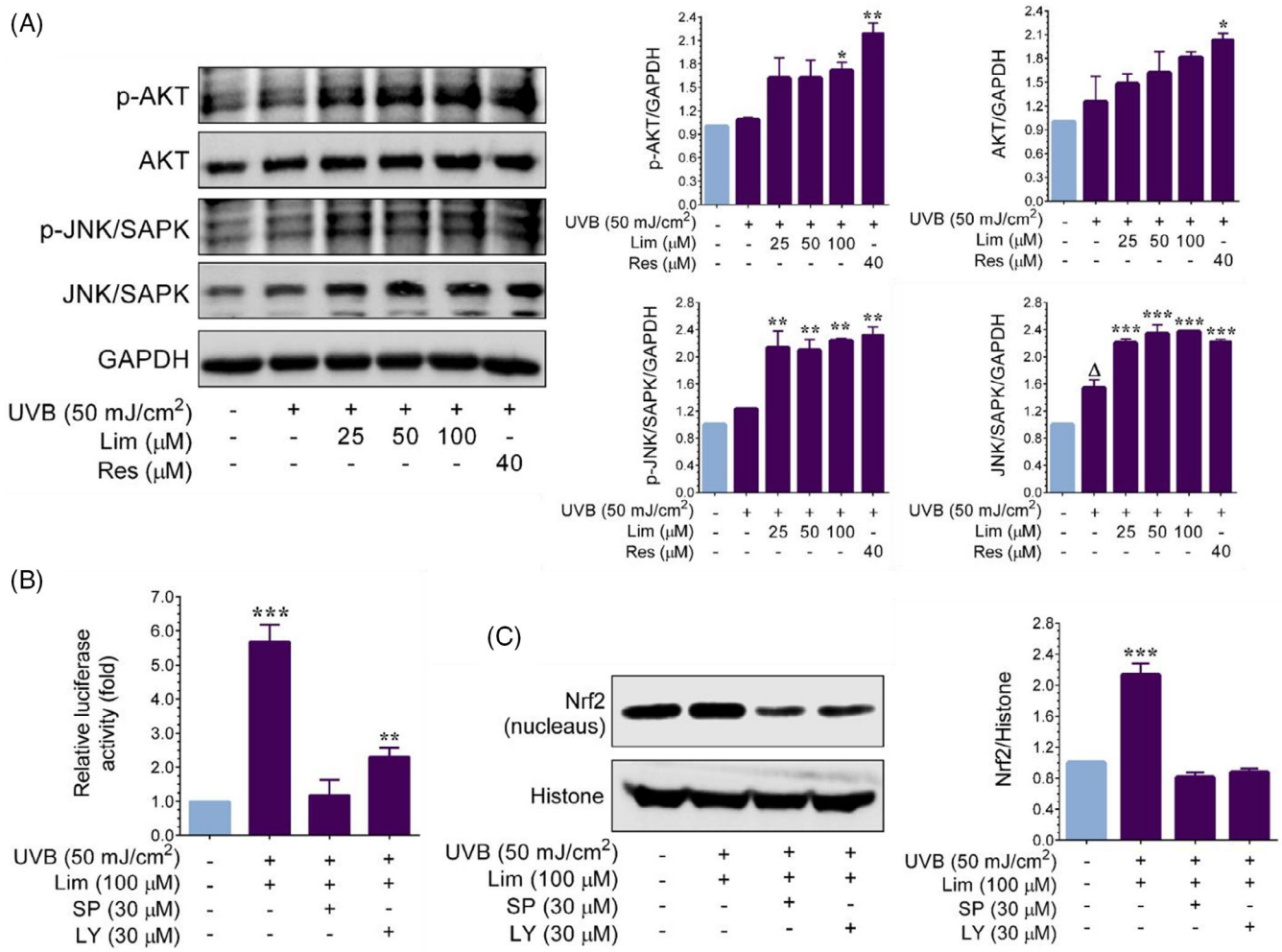

FIGURE 4 Limonene promote Nrf2 activity via activation of JNK/SAPK and AKT kinases. (A) To determine the protein expression levels of phos-AKT, AKT, phos-JNK/SAPK, and JNK/SAPK, HaCaT cells were preincubated with limonene (25–100 μM) or resveratrol (40 μM) for 2 h, and exposed to UVB (50 mJ/cm^2), and then incubated for 30 min. Total cell lysates were prepared and subjected to western blot analysis. Histogram shows the relative protein expression levels of phos-AKT, AKT, phos-JNK/SAPK, and JNK/SAPK, which are normalized with an internal control GAPDH. (B) To determine the Nrf2 transcriptional activity under pharmacological inhibition of its upstream kinases, HaCaT cells were transiently transfected with ARE promoter construct using lipofectamine and preincubated with JNK/SAPK and AKT inhibitors SP600125 and LY294002, respectively, and then further incubated with limonene (100 μM) for 2 h, and exposed to UVB (50 mJ/cm^2), and then incubated for 6 h. Relative ARE promoter activity was determined. (C) Nrf2 protein levels in the nucleus were determined by western blotting with nuclear fraction. HaCaT cells were preincubated with limonene (25–100 μM) or resveratrol (40 μM) for 2 h, and exposed to UVB (50 mJ/cm^2), and then incubated for 1 h. Histogram shows the relative protein expression level Nrf2, which are normalized with an internal control histone H3. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ compared with UVB alone versus UVB + sample treatment groups. HaCaT, human skin keratinocytes; Nrf2, NF-E2-related factor-2; UVB, ultraviolet B

pathways.[21] To determine whether limonene inhibits UVB-induced α-MSH secretion, HaCaT cells were preincubated with limonene (25–100 μM) for 2 h, and then exposed to UVB (50 mJ/cm^2) for 24 h. The intercellular levels of α-MSH was determined by a commercially available EIA kit. As shown in Figure 5A, UVB (50 mJ/cm^2) treatment significantly increased α-MSH secretion by about 14-fold (7.6 ng/ml) over 24 h, compared with non-UVB-treated cells (0.53 ng/ml). The increase was significantly as well as dose-dependently inhibited by limonene pretreatment. Limonene treatment reduced UVB-induced α-MSH secretion to 4.1, 1.4, and 0.1 ng/ml by 25, 50, and 100 μM, respectively. Indeed, compared with 100 μM GA (1.0 ng/ml), a positive drug control, a remarkable suppression was found at a dose of 100 μM limonene.

Previous data had suggested that exposure of skin keratinocytes to UVB induces POMC, a precursor of α-MSH, which further binds to melanocytes and activates melanogenesis.[10] Our data also confirm that UVB treatment significantly increased POMC mRNA level in HaCaT cells. In order to determine if the inhibition of α-MSH secretion by limonene is possibly related to POMC inhibition, we tested whether limonene inhibited UVB-induced POMC expression in HaCaT cells. As shown in Figure 5B, qPCR analysis showed that pretreatment with limonene significantly decreased UVB-induced POMC

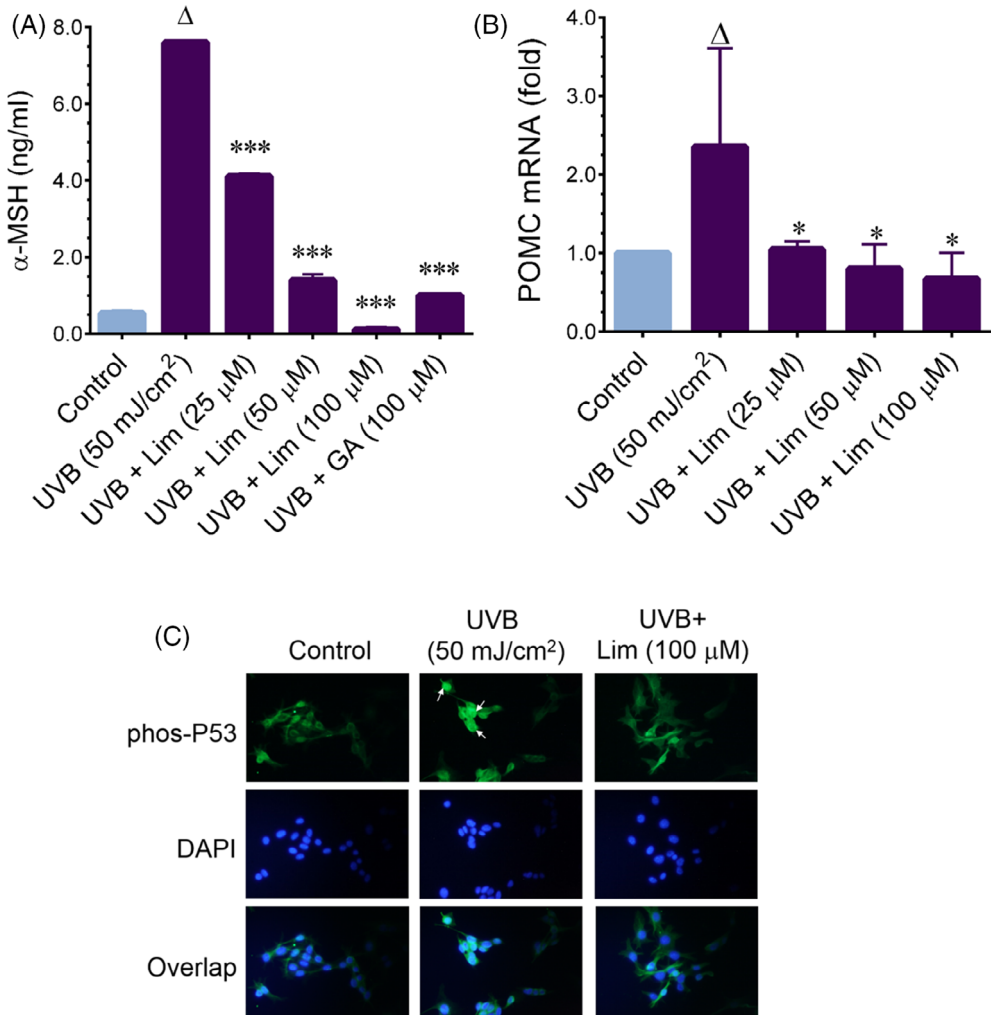

FIGURE 5 Limonene inhibits UVB-induced α-MSH secretion via suppression of p53 in HaCaT cells. (A) Intracellular α-MSH level was determined by commercially available EIA kit. (B) To quantify the mRNA expression level of POMC, HaCaT cells were preincubated with limonene (25–100 μM) and exposed to UVB (50 mJ/cm^2), and then incubated for 6 h. Total RNA was extracted and subjected to Q-PCR analysis. Relative POMC mRNA level was normalized with GAPDH mRNA. (C) The protein expression and localization of phos-p53 was measured by immunofluorescence using phos-p53 specific primary antibody with FITC-conjugated secondary antibody (green). DAPI was used to stain the nucleus. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{*}p < .05$, $^{***}p < .001$ compared with UVB alone versus UVB + sample treatment groups. HaCaT, human skin keratinocytes; POMC, proopiomelanocortin; q-PCR, quantitative-PCR; UVB, ultraviolet B; α-MSH, α-melanocyte-stimulating hormone

expression in a dose-dependent manner. These data confirm that the inhibition of α-MSH secretion by limonene is associated with the suppression of POMC in HaCaT cells.

3.6 | Limonene inhibits UVB-induced p53 activation in HaCaT cells

Previous studies have shown that p53 regulate POMC expression, and rapid induction of POMC following UVB exposure of keratinocytes is consistent with rapid activation of p53.[10] In addition, the p38 MAPK is one of the most important mediators of p53 phosphorylation at serine 389 induced by UV irradiation.[22] Therefore, we examined whether limonene inhibits the UVB-induced transcriptional activity of p53, the nuclear export of p53 was examined. Results of immunofluorescence analysis show that cells exposed to UVB led to increased p53 phosphorylation and nuclear translocation as evidenced by accumulation of phosphorylated p53 in the nucleus, while limonene pretreatment significantly inhibited UVB-induced p53 phosphorylation and nuclear translocation in HaCaT cells (Figure 5C).

3.7 | Limonene inhibits UVB-induced loss of skin barrier function in HaCaT cells

It has been well demonstrated that TJ molecules play a crucial role in the skin's barrier function. These molecules are multi-protein

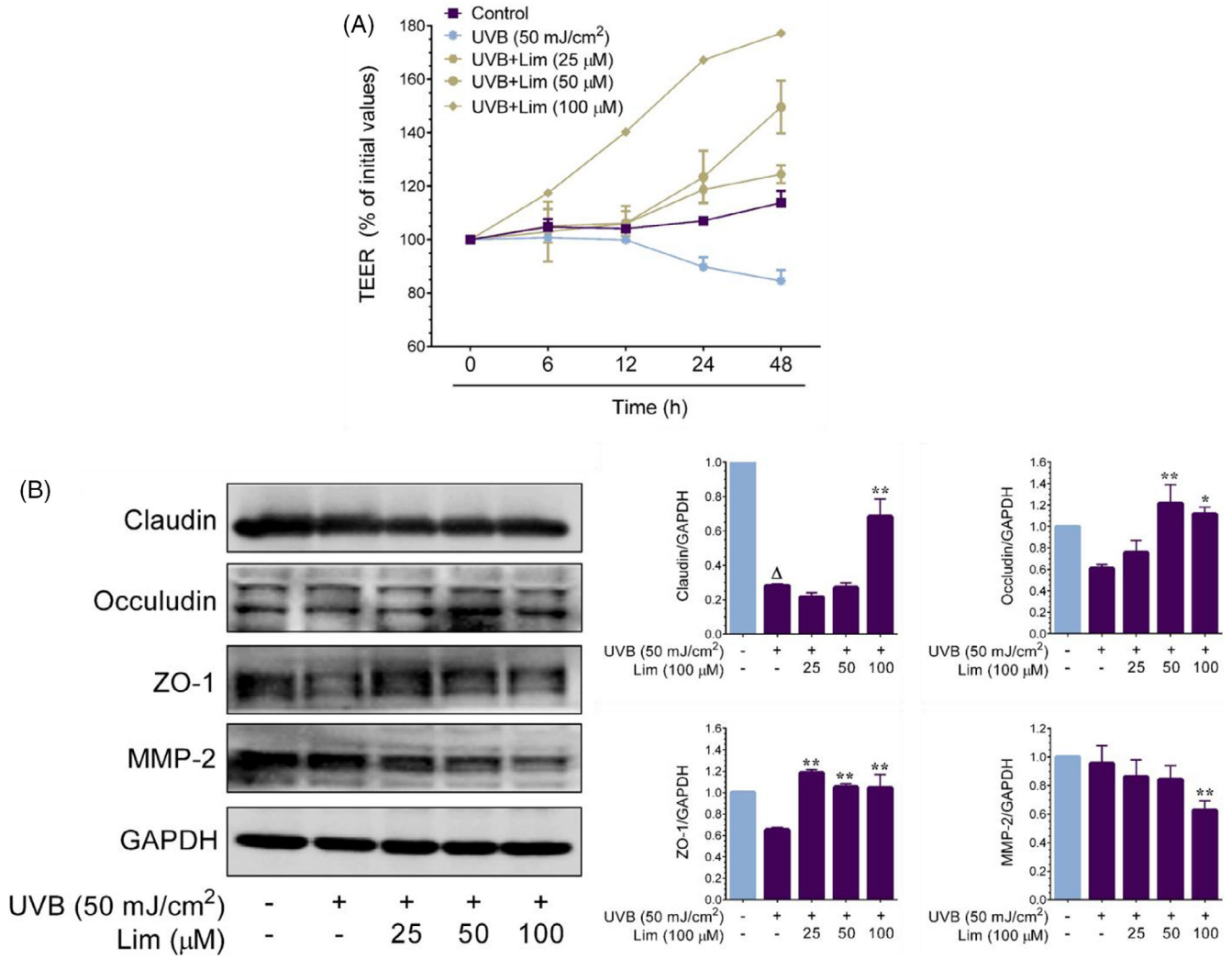

FIGURE 6 Limonene inhibits UVB-induced loss TJ regulatory proteins in HaCaT cells. (A) Change in trans-epithelial electrical resistance (TEER) across confluent HaCaT monolayers (5 days old). (B) To determine the protein expression levels of claudin, occuludin, ZO-1, and MMP-2, HaCaT cells were preincubated with limonene (25–100 μM) for 2 h and exposed to UVB (50 mJ/cm^2), and then incubated for 24 h. Total cell lysates were prepared and subjected to western blot analysis. Histogram shows the relative protein expression levels of claudin, occuludin, ZO-1, and MMP-2, which are normalized with an internal control GAPDH. Values represent the mean ± SD of three independent experiments. Statistical significance was set at $^{\Delta}p < .05$ compared to control versus UVB (50 mJ/cm^2) and $^{*}p < .05$, $^{***}p < .001$ compared with UVB alone versus UVB + sample treatment groups. HaCaT, human skin keratinocytes; TJ, tight junction; UVB, ultraviolet B

complexes, of which the main components are transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAM-A and JAM-B), and basement membrane proteins such as MMPs and uPAs.[12] Several studies have examined the effects of UVB on epidermal barrier function and have revealed that UVB disrupts this barrier.[23] To examine whether limonene could protect skin keratinocytes from UVB-induced loss of barrier function, we determined UVB-induced loss of barrier function in HaCaT cells using TEER assay. As shown in Figure 6A, compared to the untreated control group, UVB treatment markedly increased loss of barrier function as evidenced by a significant decrease of TEER. Interestingly, pretreatment with limonene significantly and dose- and time-dependently increased TEER in the UVB-induced HaCaT cells. Next, we sought to determine the effect of limonene on TJ regulatory proteins in UVB-challenged HaCaT cells. UVB irradiation resulted in a remarkable depletion of occludin and ZO-1 in HaCaT cells. Compared to the UVB-irradiated group, treatment with limonene significantly protected or recovered occludin and ZO-1 from UVB-mediated loss of expression (Figure 6B). Indeed, compared with a high dose of limonene (100 μM), 25 μM limonene exhibited excellent protection. However, claudin, another essential protein in the tight junction, was unaffected by either UVB or limonene treatment. In addition, limonene treatment significantly and dose-dependently inhibited MMP-2 levels in the UVB-irradiated HaCaT cells (Figure 6B). Accordingly, these data suggest that limonene pretreatment could protect skin keratinocytes from UVB-induced loss of skin barrier function.

4 | DISCUSSION

There is substantial evidence that UVR exposure plays a critical role in developing of several acute and chronic skin health problems, including inflammation, immunosuppression, photodamage, premature skin aging (photoaging), and skin cancers.[24] The most significant cause of skin photodamage is UV-induced oxidative stress. Oxidative stress occurs when the production of free radicals and the ability of the body to neutralize them do not match. One feasible method to overcome free radical-mediated cell damage is to increase antioxidant intake to strengthen the endogenous oxidative defense mechanism. As part of plant-rich diets, humans have been consuming sun-protecting phytochemicals for millennia, they are of low toxicity, and it is very difficult to overdose on such naturally occurring antioxidants. In recent years, research has focused on dietary antioxidants other than vitamins, such as terpenes, which scavenge free radicals and protect cells from oxidative damage. Among them, the natural monoterpenoid limonene, which is found in a variety of citrus fruits, and aromatic plants and vegetables, exhibits several biological functions, including antioxidant, antidiabetic, anticancer, anti-inflammatory, cardioprotective, gastroprotective, hepatoprotective, immune modulatory, anti-fibrotic, and anti-genotoxic effects.[16] In the present study, limonene was shown to protect HaCaT cells from UVB-induced photodamage, and the underlying molecular mechanisms of this process were investigated.

Recent studies have demonstrated anti-photoaging activity of many naturally occurring agents. Most of this activity is due to their antioxidant properties, such as terpenes found in volatile oils.[15,25] Several studies have shown that terpenes are ROS scavengers that donate hydrogen. Moreover, they exert their antioxidant activity by restoring the antioxidant system and inhibiting UVB-induced photoaging.[15] Free radical scavenging activity can be evaluated using the stable DPPH radical model, which is a commonly used and relatively quick method. DPPH radicals are thought to be scavenged by antioxidants thanks to their hydrogen-donating properties.[26] Limonene exhibited a concentration-dependent increase in its capacity to scavenge DPPH free radicals. However, the inhibition was found to be moderate, and the IC_{50} value of limonene was 392.81 μM, which is highly consistent with others observation that limonene exhibited DPPH free radical scavenging effect with an IC_{50} value of 384.73 μM.[27]

HaCaT cells have been extensively used as an in vitro model of epidermal skin to study the effects of UVB radiation.[28] In this study, we exposed HaCaT cells to increasing doses of UVB (25–100 mJ/cm^2) and found that when the UVB irradiation dose reached 50 mJ/cm^2, the HaCaT cells were significantly damaged, which contributed to the morphological change, such as cell deformation, volume reduction, karyopyknosis, and cell death. This observation correlates well with another report that 57 mJ/cm^2 UVB radiation causes cell death in HaCaT cells.[29] Also, the UVB dose used in this study was within the normal range of UV exposure, which makes these effects of interest due to their impact on human health.

Several monoterpenoids found in volatile oils are regulators of cellular redox balance. These compounds promote the cellular antioxidant defense system through induction of Nrf2-mediated antioxidant genes, including HO-1, NQO-1, and phase II enzymes.[30] There is evidence that several substances found in nature exhibit strong anti-oxidant properties that can counteract radiation-induced oxidative damage in biological systems.[31,32] A recent study has shown that linalool, a natural monoterpenoid protects human skin cells from UVB-induced oxidative stress.[33] Limonene is a natural monoterpenoid, therefore, we hypothesized that it can protect skin keratinocytes from UVB-induced photodamage. Before that, we determined the cytotoxic effect of limonene on HaCaT cells. Limonene does not cause cytotoxicity in HaCaT cells at doses up to 100 μM, but beyond that level cytotoxicity does occur. The range of nontoxic limonene concentrations was established in HaCaT cells and their ability to inhibit UVB-induced oxidative stress and cell death was investigated. As we expected, limonene significantly and dose-dependently inhibited UVB-induced ROS generation and cell death. Studies have shown that activating Nrf2 protects skin cells against UVB-irradiation induced photodamage.[34] Next, to study the possible molecular mechanisms underlying limonene's antioxidant properties, we examined the Nrf2-dependent endogenous antioxidant system in UVB-induced HaCaT cells. As UVB could not activate Nrf2 effectively, there was no difference in transcriptional activation of Nrf2 between control and UVB exposed cells. There is evidence that Nrf2 accumulates rapidly in the nucleus under-stimulation (e.g., from antioxidants), and regulates the skin's homeostasis in a cascade response. Our results showed that limonene effectively promoted Nrf2 nuclear accumulation in UVB-treated HaCaT cells, and the levels of nuclear accumulation of Nrf2 in HaCaT cells were higher after UVB irradiation compared to untreated cells. Furthermore, to counteract UVB-induced oxidative stress damage, endogenous antioxidant cytoprotective proteins, such as HO-1 and NQO-1were significantly increased by limonene. Additionally, limonene reversed the down-regulated γ-GCLC in UVB-induced HaCaT cells. The effect of Nrf2 was further confirmed by interfering with the Nrf2 expression in HaCaT cells. Our results showed that UVB irradiation significantly increased ROS generation in siNrf2 transfected cells compared to untreated control cells, while limonene failed to protect UVB-induced ROS generation in siRNA-mediated Nrf2 knock-down cells. According to Surh,[7] during oxidative stress conditions, the activation of the Nrf2 cascade was critically regulated by their upstream regulators such as JNK/SAPK, p38 MAPK, ERK1/2, and AKT/PI3K. Therefore, we monitored the expression of MAPKs proteins (JNK/SAPK, ERK1/2, and p38 MAPK) and AKT/PI3K under the same experimental conditions. We found that limonene significantly, and dose-dependently activates JNK/SAPK and AKT kinases; yet, on the other hand, pharmacological inhibition of these kinases reduced limonene-mediated Nrf2 activity. Thus, we suggest that the activation of Nrf2 by limonene is a JNK/SAPK- and AKT-dependent mechanism. A previous study also suggested that exposure of HaCaT cells to dietary phytochemicals, such as hyperoside and quercitrin promote Nrf2 accompanied by the activation of JNK/SAPK and AKT.[35] Based on our results, limonene promoted Nrf2 activation and induction of antioxidant regulators, which were critical protective molecules for the skin against UVB-induced photodamage.

It was previously demonstrated that HaCaT cells were exhibited morphological changes in response to UVB exposure, as a result, irregularities in keratinocyte volume may disrupt the organization of TJs. Several TJ proteins, such as occludin and ZO-1, frequently lose their function during photoaging.[14] Studies also suggest that ROS generated in response to UVB exposure are responsible for the loss of either skin barrier function or TJ molecules.[14] Thus, we hypothesize that the antioxidant activity of limonene could prevent UVB-induced loss of barrier function in keratinocytes. TEER is a functional parameter to monitor the quality of epithelial cells in vitro. Recently, the TEER assay was established as a screening parameter to determine skin epithelial barrier function.[36] We found that UVB treatment significantly decreases the TEER of HaCaT cells, while pretreatment with limonene significantly as well dose-dependently, increased the TEER value. Additionally, limonene prevents UVB-induced depletion of occludin and ZO-1 expression in HaCaT cells.

During UVB-induced photodamage, the increase of MMPs destroys the original structure of the extracellular matrix (ECM) and causes various adverse reactions in the skin. In order to protect skin from photodamage, it may be necessary to inhibit UVB-induced MMPs expression. MMP-2, a gelatinase, can specifically degrade the most abundant type I & IV collagen in the ECM.[37] In this study, limonene was found to inhibit UVB-induced MMP-2 expression in HaCaT cells. Several studies have documented that NF-κB is an inflammation-related heterodimer that consists of p50 and p65 subunits, which promote MMP-2 activity in human skin cells.[38] A previous study showed that limonene significantly inhibits NF-κB activity in lung cells.[39] This indicates that limonene-mediated MPP-2 reduction was possibly through suppression of NF-κB activity.

5 | CONCLUSION

The present study provides experimental evidence that limonene protects keratinocytes from UVB-induced photodamage and photoaging by blocking intracellular ROS generation as well as cell death. Our mechanistic study demonstrates that UVB-induced oxidative stress and cell death of keratinocytes are prevented by up-regulation of Nrf2-dependent endogenous antioxidants, especially HO-1, NQO-1, and γ-GCLC. Our study further suggests that Nrf2 activation by limonene may represent a promising intervention in UVB-induced photodamage as well as photoaging. Additionally, limonene protects keratinocytes from UVB-mediated loss of skin barrier function and secretion of pro-melanogenic factors. Indeed, limonene is useful for preventing photodamage in people whose skin is exposed to daily doses of UVB radiation such as those who work outdoors, thus enabling their skin to maintain its integrity and barrier function. Our findings suggest that limonene may serve as a novel source for developing dermato-protective agents for cosmetic applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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