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Protective effects of resveratrol on UVB-irradiated HaCaT cells through attenuation of the caspase pathway

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Abstract. Resveratrol, a polyphenol present in grapes and red wine, exerts wide biological properties including antioxidant, chemoprevention and cardioprotective effects. It was recently reported that resveratrol attenuates the oxidative DNA damage in neuronal cells. However, whether resveratrol attenuates the UVB-induced cell death in skin still remains largely unknown. In this study, to confirm the photoprotection properties of resveratrol, we investigated the effect of the pretreatment of resveratrol on UVB-treated HaCaT cells and molecular mechanisms concerning apoptotic pathways. Our data showed that HaCaT cells markedly decreased cell proliferation rates after UVB irradiation. Notably, the pretreatment of resveratrol on UVB irradiation resulted in an increase in cell survival which concomitantly reduced the reactive oxygen species production. Furthermore, the activation of caspase-3 and -8 was partially decreased in resveratrol-pretreated HaCaT cells, implying that the attenuation of caspase-3 and -8 activation is involved in cell survival after UVB irradiation. Thus, our data indicate that resveratrol may be a promising photoprotection agent, used in sunscreen products, to reduce cell death in UV-damaged skin.

Introduction

Ultraviolet B (UVB) is a well-known major risk factor for the development of acute inflammation, photoaging as well as non-melanoma skin cancer on the epidermis (1,2). Many studies clearly indicate that UVB exerts its detrimental effect mainly through the induction of direct DNA damage or the production of reactive oxygen species (ROS) (3-5). Thus, it may be a fundamental step in the prevention of skin cancer by reducing ROS production. Photochemoprotection using antioxidants has been tried and suggests that it may reduce the adverse effects of UVB on the skin (6-8).

Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenolic phytoalexin found in the skin and seeds of grapes, nuts, fruits and red wine (7,8). Resveratrol is a potent antioxidant with anti-inflammatory and anti-proliferative properties (9,10). Notably, resveratrol inhibited UVB-induced skin edema and the production of hydrogen peroxide in SKH-1 hairless mice (11). In another study, the pretreatment of human keratinocytes with resveratrol inhibited the UVB-induced activation of the NF-κB pathway (12). Furthermore, it was reported that resveratrol prevents UVB-induced radiation damage in mouse skin through the modulation of survivin (13). However, whether the photochemoprotective effects of resveratrol on the human keratinocyte cell line, HaCaT cells, on UVB induced-cell death, are mediated by the modulation of the caspase pathway is largely unknown.

To elucidate the photochemoprotective pathways of resveratrol in the skin on UVB irradiation, especially focusing on caspase activation, we investigated the caspase pathway in UVB-irradiated HaCaT cells with or without resveratrol.

Material and methods

Materials. Antibodies against tubulin, Bcl2, $Bcl_{X/L}$, Bax, PARP, caspase-3, -8 and -9 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture. Human keratinocyte cell line, HaCaT cells, were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in a culture dish and maintained in the tissue culture incubator. Cells were preincubated with resveratrol for 30 min prior to UVB irradiation at 200 mJ/cm^2.

UVB irradiation. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 38 cm. The energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer, International Light,

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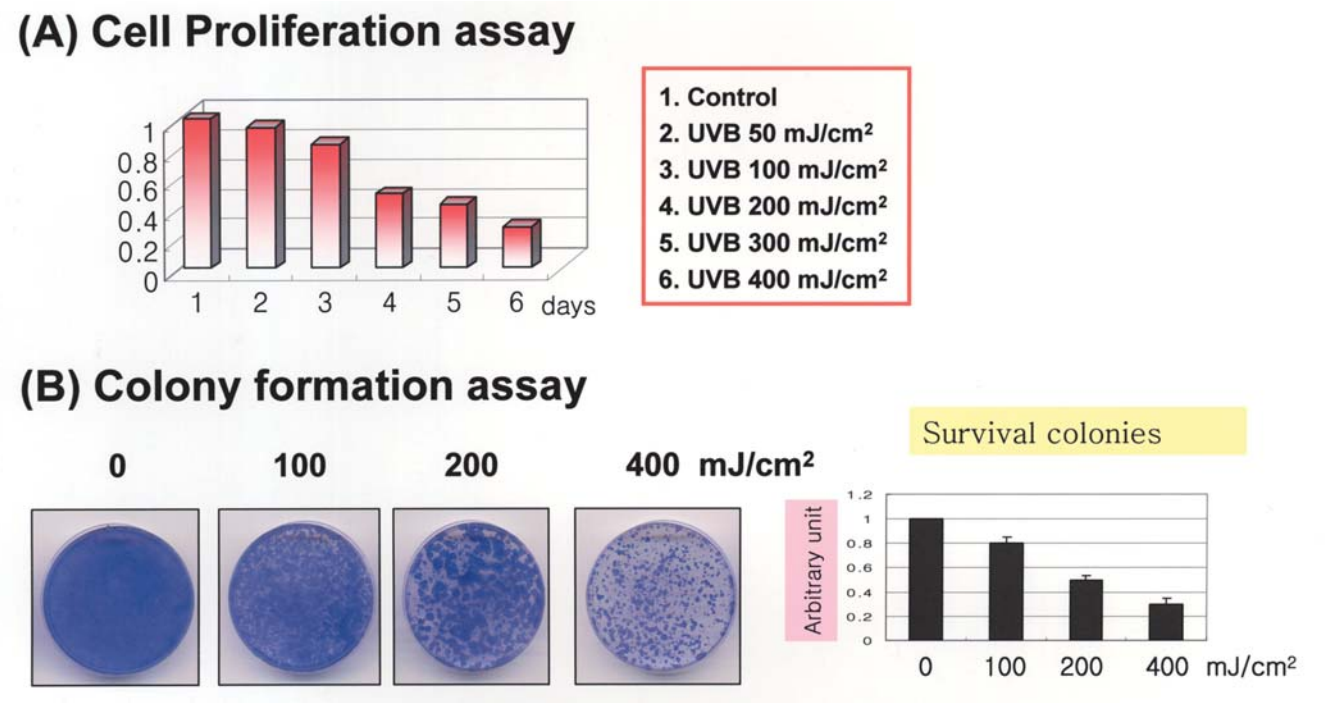

Figure 1. Decreased cell proliferation rate in UVB-irradiated HaCaT cells. Cells were treated with various doses of UVB using seven Westinghouse FS-40 sunlamps for 6 days. The viable cells were counted daily using a trypan blue assay (A). Survival colonies were also stained with crystal violet staining solution (B). Experiments were repeated two times and similar results were obtained.

Newburyport, MA). Cells were exposed for 0 and 360 sec of UVB, corresponding to doses of 0 and 400 mJ/cm^2. To prevent light absorption by a tissue-culture medium, the culture medium was removed just prior to irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells. After UVB irradiation, cells were fed with a fresh growth medium.

Western blot analysis. Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline and 28 mM benzamidine-HCl] for 30 min on ice. Lysates were clarified by centrifugation and quantified using the Bradford assay (Life Science Co., CA, USA) with bovine serum albumin as a reference standard. Proteins (35 μg) were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to immobilon-P transfer membrane (Millipore Co., MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science Co., Buckinghamshire, UK).

Measurement of the caspase activities. Cells were collected, washed in PBS and re-suspended in a buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$ 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin]. Cell lysates were clarified by centrifugation at 12,000 rpm for 10 min and 50 μg of protein of the supernatant was incubated with 50 μM Ac-DEVD-*p*Na colorimetric substrate at 37˚C for 1 h. The optical density was measured at 405 nm.

Measurement of the reactive oxygen species (ROS) generation. The generation of ROS was measured by a flow cytometry analysis using DCFH-DA as a substrate. Briefly, after treatment, cells were harvested, washed twice with PBS and suspended in PBS (1x10^6 cells/ml). The cell suspension (500 μl) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM and incubated at 37˚C for 15 min. Cells were then irradiated with UVB and the ROS generation was assessed by the DCF fluorescence intensity (FL-1, 530 nm) from 10,000 cells with a FACS Calibur flow cytometer (Becton-Dickinson).

Results

UVB induces decreased cell proliferation rates in HaCaT cells. To study the effect of UVB on cell proliferation rates, HaCaT cells were exposed to UVB at doses ranging from 50 to 400 mJ/cm^2 and then the viable cells were counted using the trypan blue exclusion method. As shown in Fig. 1A, the number of viable cells was decreased in a dose-dependent manner of UVB irradiation. The proliferation rates of UVB-irradiated HaCaT cells on a 200 mJ/cm^2 dose were decreased ~50% compared to the non-irradiated cells. The survival colonies were stained with crystal violet 72 h after UVB irradiation and the pattern of the stained colonies (Fig. 1B) was similar to the viable cell numbers as shown in Fig. 1A.

Attenuation of the ROS generation on UVB-irradiated HaCaT cells by resveratrol. To determine the inhibitory effect of resveratrol on the generation of ROS in UVB-irradiated HaCaT cells, the cells were pretreated with resveratrol and the amount of ROS in HaCaT cells was assessed by the DCF fluorescence intensity (FL-1, 530 nm) with a FACS Calibur

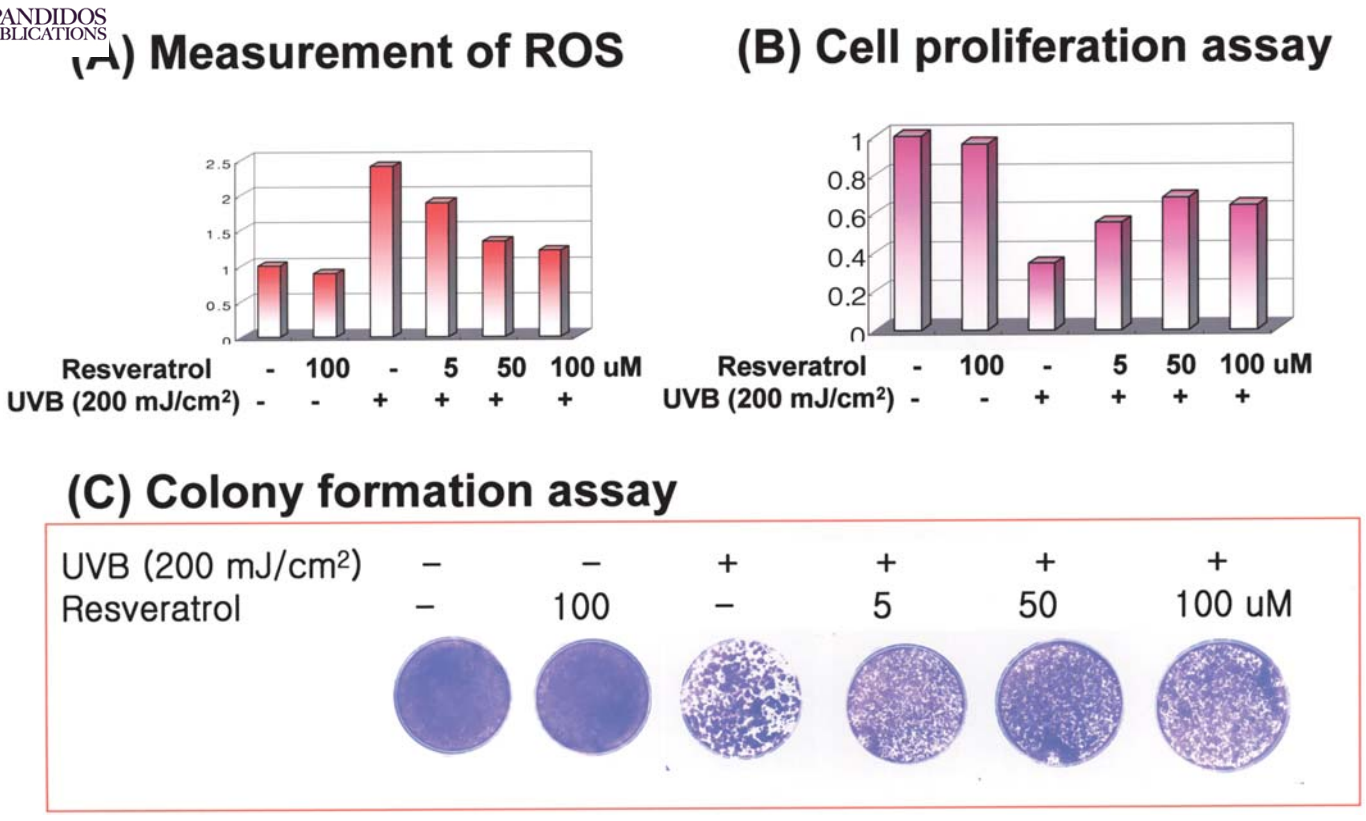

Figure 2. A decreased ROS production partially restored the survival colonies in resveratrol-treated HaCaT cells. The generation of ROS (A) and cell proliferation rates (B) were measured in resveratrol-treated cells after UVB irradiation. The survival colonies were also stained with crystal violet staining solution in resveratrol-treated cells after UVB irradiation (C).

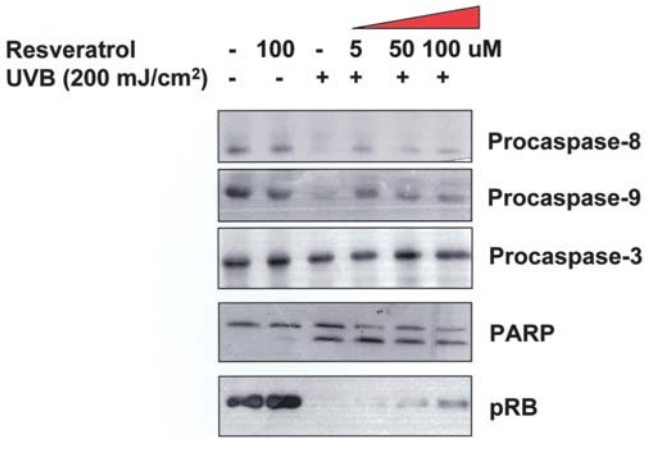

Figure 3. Attenuation of the activation of caspase proteins in resveratrol-treated cells after UVB irradiation. Cells were preincubated with various concentrations of resveratrol for 30 min and then exposed to UVB (200 mJ/cm^2). Total proteins were extracted and subjected to immunoblotting using anti-caspase-9, -8 and -3, Rb and PARP antibodies. The data are representative of two independent experiments.

flow cytometer. The generation of ROS was attenuated in resveratrol-pretreated HaCaT cells after UVB irradiation, implying that the roles of resveratrol attenuated the generation of ROS in UVB-damaged cells (Fig. 2A). Notably, the pattern of cell proliferation rates was similar to the amount of ROS in the cells (Fig. 2B). The survival colonies were also increased in the resveratrol-treated cells after UVB irradiation (Fig. 2C).

A *decreased caspase activation of UVB-irradiated HaCaT cells by resveratrol*. To study the effect of resveratrol on the caspase pathway in UVB-damaged cells, we investigated the expression of caspase-3, -8 and -9, PARP and the Rb protein in resveratrol-pretreated HaCaT cells after UVB irradiation. When HaCaT cells were irradiated with UVB (200 mJ/cm^2), the activation of caspase-8 and -9, evidenced by a decreased expression of procaspase-8 and -9 was clearly observed, but not in caspase-3 (Fig. 3). In addition, PARP and pRb cleavages were detected in UVB-irradiated HaCaT cells (Fig. 3). Of note are the levels of procaspase-8 and -9, PARP and Rb which were partially restored in a dose-dependent manner of resveratrol concentration, implying that UVB-damaged cells are protected from ROS by a pretreatment of resveratrol and this event is partially mediated by inhibition of the caspase activation.

To confirm whether the caspase-3 is activated in UVB (200 mJ/cm^2)-irradiated HaCaT cells, we measured the caspase-3 activation using an Ac-DEVD-*p*Na colorimetric substrate. According to the Ac-DEVD-*p*Na colorimetric assay, caspase-3 was activated in UVB-irradiated HaCaT cells and the activity was partially attenuated by the pretreatment of resveratrol (Fig. 4).

Then, we studied the expression of Bcl2, $Bcl_{X/L}$ and Bax expression in UVB-irradiated HaCaT cells. The expression

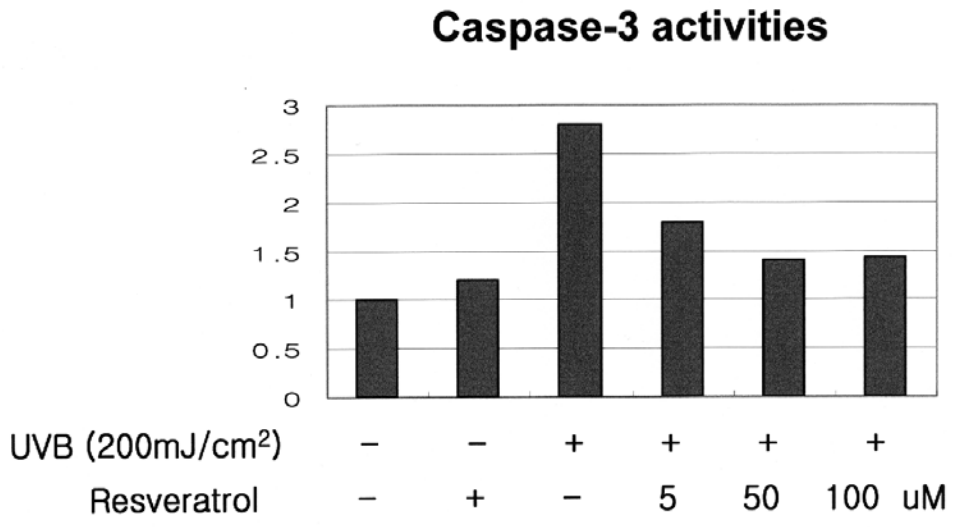

Figure 4. Decreased activation of caspase-3 in resveratrol-treated HaCaT cells after UVB irradiation. Cells were preincubated with various concentrations of resveratrol for 30 min and then exposed to UVB (200 mJ/cm^2). The activity of caspase-3 was measured using Ac-DEVD-*p*Na according to the manufacturer's instructions.

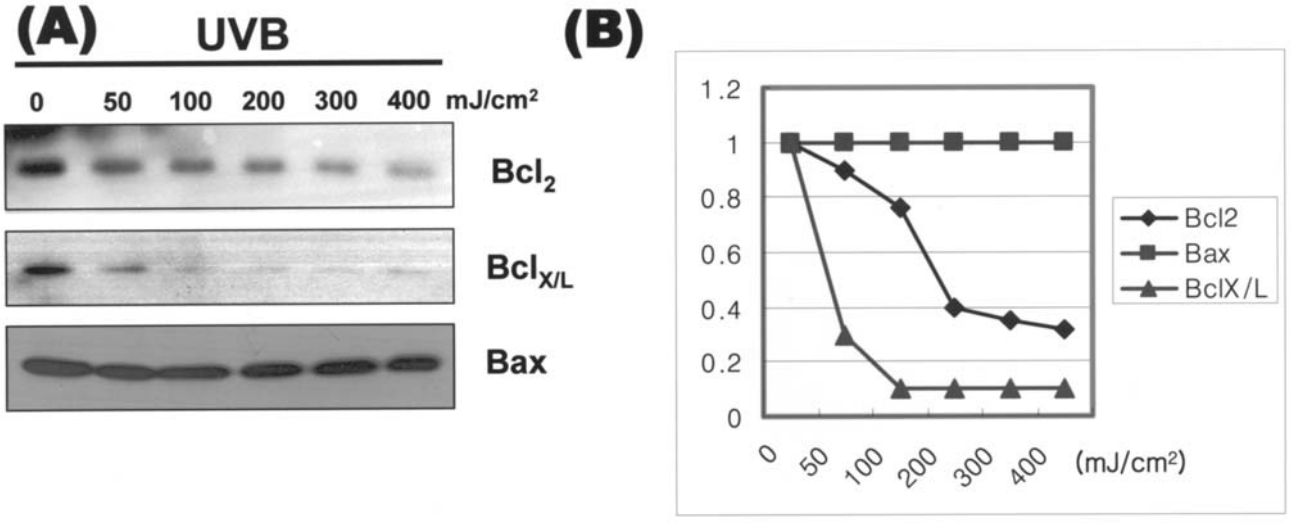

Figure 5. Expressions of the Bcl2 family proteins in UVB-irradiated cells. Cells were exposed to various doses of UVB (50-200 mJ/cm^2). Total proteins were extracted and subjected to immunoblotting using anti-Bcl2, $Bcl_{X/L}$ and Bax antibodies. The data are representative of two independent experiments.

of Bcl2 and $Bcl_{X/L}$ was markedly decreased in UVB-irradiated HaCaT cells (Fig. 5). However, the decreased expression of Bcl2 and $Bcl_{X/L}$ was not attenuated by resveratrol in UVB-irradiated HaCaT cells (data not shown).

Discussion

Skin cancer, including squamous and basal cell carcinoma, occur primarily on sun-exposed areas of the body (14,15). The main causative agent is chronic sun exposure. Besides using sunscreen agents, additional efforts are needed to prevent skin cancers.

It was recently reported that several botanical agents such as curcumin, resveratrol and genistein, exert anti-inflammatory, cancer-preventive, anti-photoaging and photochemoprotective effects on the skin (7,8). The prevention of UV-induced cancer or photochemoprotection is mainly mediated by reducing the ROS generation, resulting in the attenuation of UV-induced DNA damage (14,16,17). Thus, the photochemoprotective agents for human use should have the ability to ameliorate the adverse biological effects of UV radiation, such as excessive ROS generation and DNA mutation.

In this study, our data clearly show that resveratrol partially inhibits UVB-induced cell death through a decreased production of ROS generation and inhibition of caspase pathways, but not in the Bcl2 family proteins.

Resveratrol is a potent antioxidant with anti-inflammatory and anti-proliferative properties (8). We recently reported that UVB irradiation induces decreased cell proliferation rates of HaCaT cells, evidenced by DNA fragmentations, caspase-3 and -8 activation, in a concentration-dependent

SPANDIDOS PUBLICATIONS18). In addition, the down-regulation of Bcl2 and re clearly observed in UVB-irradiated HaCaT cells. In this study, the UVB-induced generation of ROS was decreased by the pretreatment of resveratrol. Moreover, the clear reductions of procaspase-8 and -9 were partially restored in the pretreatment with resveratrol in UVB-irradiated HaCaT cells, suggesting that resveratrol inhibits UVB-induced cell death through a decreased ROS production and inhibition of caspase-8, -9 and -3 activation.

Consistent with our data, Jang *et al* reported that resveratrol shows the protective effect on ß-amyloid-induced oxidative PC12 cell death through the inhibition of caspase-3 (19). Shakibaei *et al* reported that resveratrol inhibits IL-1 ß-induced stimulation of caspase-3 and cleavage of PARP in human articular chondrocytes *in vitro* (20). Furthermore, resveratrol inhibits cell proliferation and prevents the oxidative DNA damage in rat fibroblasts (21). Accumulating data indicate that the possible molecular mechanisms of resveratrol exerting photochemoprotection are mediated by inhibiting the UVB-induced activation of NF-κB, modulation of mitogen-activated protein kinases and phosphorylation status of survivin (10,12,22).

Thus, our data showed that resveratrol attenuates UVB-induced cell death by inhibition of the caspase in the HaCaT keratinocyte cell line, implying that it may be developed as a promising photochemoprotective agent for the protection of UVB-induced skin damage.

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