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Protective Effect of Resveratrol on the Oxidative Stress-Induced Inhibition of Gap Junctional Intercellular Communication in HaCaT Keratinocytes

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Abstract – The aim of this study was to investigate the effect of resveratrol on the oxidative stress-induced inhibition of gap junctional intercellular communication in HaCaT keratinocytes. Anti-oxidative activity of resveratrol was measured by α,α-diphenyl-β-picrylhydrazyl assay and dichlorodihydrofluorescein diacetate oxidation assay. Gap junctional intercellular communication in HaCaT keratinocytes was assessed using the scrape loading/dye transfer technique. Western blots and reverse transcription-polymerase chain reaction were also analyzed for connexin 43 protein and mRNA expression, respectively. Resveratrol scavenged directly the stable α,α-diphenyl-β-picrylhydrazyl radical over a concentration range of 4 mg/ml (78.2 ± 2.7% of control) to 500 mg/ml (29.9 ± 4.2% of control) and decreased the intracellular reactive oxygen species induced by ultraviolet A (UVA) irradiation (89.3 ± 1.1% of UVA group), ultraviolet B (UVB) irradiation (70.9 ± 1.7% of UVB group) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 48.3 ± 1.1% of TPA group), respectively. UVA irradiation and TPA markedly reduced gap junctional intercellular communication, which was restored by resveratrol. There were no significant differences in the level of connexin 43 protein and mRNA expression among any of the experimental groups. Our data suggests that resveratrol has the protective effect on the oxidative stress-induced inhibition of gap junctional intercellular communication in HaCaT keratinocytes, and this protection is likely due to the scavenging of reactive oxygen species.

Keywords □ Resveratrol, 12-*O*-Tetradecanoylphorbol-13-acetate, Ultraviolet irradiation, Oxidative stress, Gap junctional intercellular communication, Connexin 43

INTRODUCTION

It is well accepted that excessive exposure to oxidative stresses such as high metabolic demands and outside forces like sunlight, smoking, and pollution can result in acute and chronic damage of skin. The resulting oxidative stress can damage cellular components and change the pattern of gene expression, leading to photoaging and serious skin disease such as cancer (Pinnell, 2003; Provost *et al.*, 2003).

The intercellular signaling system mediated by connexin channels is one of crucial for maintaining tissue homeostasis, growth control, development, and synchronized response of cells to various stimuli (Richard, 2000). Skin possesses an abundance of connexins (Cxs) resulting in an elaborate gap junctional network characterized by overlapping expression and functional diversity. Even though the role of gap junctions in epidermal homeostasis remains to be demonstrated, it is assumed that gap junctional intercellular communication (GJIC) is involved in regulation of keratinocyte growth and differentiation especially (Wiszniewski *et al.*, 2000). Recently, it has been reported that ultraviolet A (UVA) irradiation transiently disrupts GJIC in human keratinocytes (Provost *et al.*, 2003) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a well-known tumor promoter, inhibits GJIC (Kang *et al.*, 2000). Indeed, ultraviolet irradiation and TPA enhance the generation reactive oxygen species (ROS) and decrease the ROS detoxification enzymes in both epidermal and inflammatory cells (Nakamura, 2000; Kang *et al.*, 2001).

3,5,4'-trihydroxy-*trans*-stilbene (resveratrol) is a naturally occurring stilbene found in grape skins and red wines as a result of being generated by *Vitis vinifera* L. (Vitaceae) in response to

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fungal infection (Langcake and Pryce, 1976). It has been reported that resveratrol possesses a wide range of biological and pharmacological activities including antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic effects (Soleas *et al.*, 1997; Jang *et al.*, 1997; Surh, 1999). In light of the antioxidant properties of resveratrol, this compound may exert cytoprotective effects through regulation of cellular homeostasis that is vulnerable to oxidative stress. However, the precise mechanism of resveratrol on the cellular homeostasis remains unclear.

Therefore this study was designed to investigate the effect that resveratrol has on the GJIC, particularly oxidative stress-induced inhibition of GJIC in HaCaT keratinocytes.

MATERIALS AND METHODS

Materials

12-*O*-Tetradecanoylphorbol-13-acetate (TPA), lucifer yellow CH, resveratrol, α,α-diphenyl-β-picrylhydrazyl (DPPH), Hanks' balanced salt solution (HBSS), phosphate buffered saline (PBS) and polyclonal antibody for Cx43 were purchased from Sigma Chemical Co. (USA). All other chemicals used were of reagent grades and were locally and commercially available. TPA was dissolved in absolute ethanol (EtOH). Resveratrol was dissolved in EtOH for DPPH assay and in dimethyl sulfoxide (DMSO) for the treatment to cells. The final DMSO concentrations did not significantly affect the results.

Cell culture

HaCaT keratinocytes cell line was obtained from Dr. Fusenig (German Cancer Research Center, Division of Differentiation and Carcinogenesis In Vitro) and cultured in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum and 1% antibiotic and antimycotic solution. HaCaT cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

α,α-Diphenyl-β-picrylhydrazyl (DPPH) assay

EtOH solutions of resveratrol at various concentrations (10 μl) were added to a solution of DPPH (100 mM) in EtOH (190 μl), and the reaction mixture (total volume, 200 ml) was shaken gently. After keeping at 37°C for 30 min, the remaining DPPH was determined by colorimetry at 540 nm, and the radical-scavenging activity of each concentrations was expressed using the ratio of the absorption of DPPH (%) relative to the control DPPH solution (100%) in the absence of sample. The EC50 is the concentration required for 50% inhibition.

Dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation assay

DCFH-DA oxidation assay is based on the ability of non-polar, non-fluorescent DCFH-DA to diffuse through the cell membrane and to be deacetylated by cytosolic esterases to form the polar, non-fluorescent dichlorodihydrofluorescein (DCFH). This last is trapped inside the cell where, by reacting with intracellular reactive oxygen species (ROS), it gives rise to the formation of the fluorescent derivative dichlorofluorescein (DCF) (Reid *et al.*, 2001). For experiments, HaCaT cells were seeded at a density of 2×10^4 cells/well in 96 well plates (Nunc^{TM}, NY, USA) and incubated overnight at 37°C/5% CO_2. HaCaT cells were pre-treated for 24 h with 10 μM of resveratrol again. DCFH-DA (Molecular Probes, Inc., USA) was prepared at a concentration of 20 μM in Hanks' balanced salt solution (HBSS) for 20 min, and washed twice with HBSS and 100 μl/well HBSS added to HaCaT cells with and without resveratrol. The oxidation of DCF was measured in a Wallac Victor2 (Perkin Elmer Life Science, Turku, Finland), readings were taken at 485 ± 10 nm excitation and 530 ± 15 nm emission. 10 J/cm^2 of UVA irradiation was provided by high-intensity UVA source (Dermlight cube 401 equipped with UVA filters) emitting wavelengths of the 320~400 nm range at a distance of 40 cm. The amount of UVA administered was assessed with UVA meter (UVATEC, Inc., Sherman Oaks, CA). UVB irradiation was provided by a bank of Sankyo Denki G15T8E, a fluorescent bulb emitting 280~320 nm wave with a peak at 313 nm. UVB irradiation was delivered with the dose of 30 mJ/cm^2 and the irradiance was monitored with an IL1700 radiometer (International Light Inc., Newburyport, MA, USA). Fluorescent intensity was measured at 3 h after oxidative stress. Oxidation ratio was expressed using the ratio of the fluorescent intensity of resveratrol group (%) relative to the control group (100%) in the absence of sample.

Scrape-loading/dye transfer (SL/DT) assay

GJIC was assessed using the SL/DT technique (Kang *et al.*, 2000). The SL/DT assay utilized confluent cultures grown in 60 mm tissue culture plates. HaCaT cells were seeded at a density of 1.5×10^6 cells in 60 mm tissue culture plates and incubated overnight at 37°C/5% CO_2. HaCaT cells were pre-treated for 2 h before experiments with 1 μM of resveratrol. Then washed twice with 1X phosphate buffered saline (PBS) and 1 ml of PBS added to the cells for UV irradiation (same as

described above). After UV irradiation, fresh DMEM medium added to the cells with and without resveratrol and incubated for 2 h. DMEM containing 10 ng/ml of TPA added to the cells of TPA group, then incubated for 1 h. Treated HaCaT cells were rinsed and scraped with surgical blade. Cells were incubated with 2 ml of 0.05% lucifer yellow for 10 min and fixed with 3.7% formaldehyde PBS solution. The distance that lucifer yellow had traveled through gap junctions was observed with a fluorescent microscope. All the experiments were repeated at least three times separately to confirm their reproducibility and data from one representative experiment was used for figures.

Western blot immunoassay

HaCaT cells were seeded and treated as for the scrape loading experiments. Following exposure as indicated, plates were washed with PBS. Treated cells were harvested with lysis buffer (Cell Signaling Technology, Inc., USA) containing 1 mM phenymethylsulfonyl fluoride (PMSF) and centrifuged at 4°C 14,000 rpm for 15 min to remove insoluble materials. Protein concentration was determined with BCA protein assay kit (Pierce Biotechnology Inc., USA). Proteins were separated by 10% polyacrylamide gels and transferred to nitrocellulose membrane using a semi-dry transfer process. Bands were immunologically detected using a polyclonal antibody against human Cx 43 (Sigma Chemical Co., USA). The blotting membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:5,000) and blots were developed with the ECL enhanced chemiluminescence detection kit (Amersham, Little Chalfton, UK). The intensity of the immunoreactive bands was determined by scanning with a densitometric analysis program (Imager IIITM image analysis system, Bioneer, Daejeon, Korea).

RNA extraction and polymerase chain reaction

The total RNA was extracted with Trizol reagent (Gibco Laboratories, USA) according to the method described by the manufacturer, and then RNA extract was stored at -75°C until use. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using One Step RNA PCR kit (Takara, Shiga, Japan) according to the method described by the manufacturer using GeneAmp 9600 thermocycler (Perkin Elmer, Norwalk, USA). PCR primers were produced by custom oligonucleotide synthesis service (Bioneer, Korea). Primers used to amplify a 400-base pair (bp) fragment of the Cx 43 gene were TCTGAG TGCCTGAACTTGC (sense) and TTATCTCAATCTGCTTC AAGTGC (antisense). Target gene mRNA levels were quantified based on standard and normalized to GAPDH (control) mRNA level. Electrophoresis for the PCR products was performed in a 1.0% agarose gel and the densitometry was carried out using a scanning with a densitometric analysis program.

Statistical analysis

Overall significance was tested by one-way ANOVA. Differences between groups were considered significant at $P<0.05$. All results are presented as means ± SEM.

RESULTS

Antioxidant effect of resveratrol

Resveratrol was shown to scavenge directly the stable DPPH radical over a concentration range of 4 mg/ml (78.2 ± 2.7% of control) to 500 mg/ml (29.9 ± 4.2% of control). It scavenged the stable DPPH radical in a concentration-dependent manner. The EC50 value of resveratrol calculated to be 28.1 mg/ml. The EC50 value of trolox (vitamin E analog), as positive control, was 10.3 mg/ml (Fig. 1). Intracellular reactive oxygen species (ROS) were measured using DCFH-DA oxidation assay. Intracellular ROS level of resveratrol group was decreased to 89.3 ± 1.1% compared to 10 J/cm^2 of UVA irradiation group. Resveratrol significantly reduced the intracellular ROS level after UVB treatment (70.9 ± 1.7% compared to UVB irradiation group) and TPA treatment (48.3 ± 1.1% compared to TPA group) (Fig. 2).

Effect of resveratrol on gap junctional intercellular communication (GJIC)

The GJIC was assessed using the SL/DT assay. After exposing the cells to TPA for 1 h, an apparent decrease in GJIC was detected at a dose level of 10 ng/ml (Fig. 3A, B). This decrease

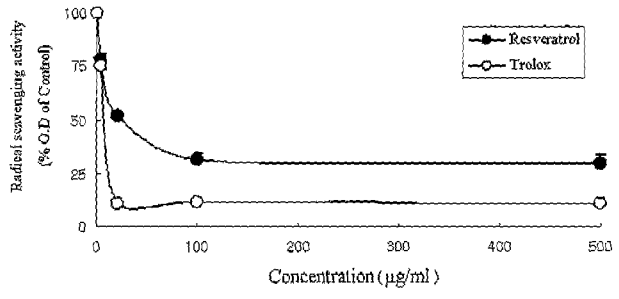

Fig. 1. Effect of resveratrol on α,α-diphenyl-β-picrylhydrazyl assay. Each value is the mean ± SEM of 6 repetitions per group.

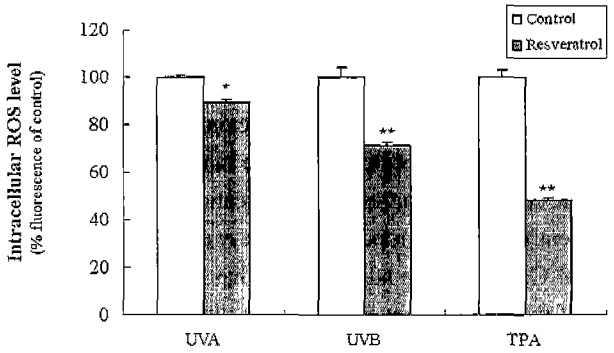

Fig. 2. Effect of resveratrol on DCF oxidation in HaCaT keratinocytes. Each value is the mean ± SEM of 6 repetitions per group. *,**Significantly different($p<0.05$, $p<0.01$) from control.

was significantly prevented by resveratrol (Fig. 3C). Similar to TPA two hours after the cells exposed to UVA irradiation, GJIC was decreased (Fig. 4A, B). This decrease was significantly prevented by resveratrol (Fig. 4C). In contrast, the inhibition of GJIC induced by UVB was not prevented by resveratrol treatment (data not shown).

Effect of resveratrol on the protein and gene expression of Cx 43

As shown in Fig. 5 and 6, the levels of Cx 43 protein ratio and mRNA expression ratio were 0.93 ± 0.05 and 0.70 ± 0.06 in control cells, respectively. Cx 43 protein was shown in two forms phosphorylated (46 kDa) and unphosphorylated (43 kDa) (Fig. 5). There were no significant changes in the protein and gene expression of Cx 43 of UVA- or TPA-treated cells compared with those of control values. Both UVA and TPA changed the proportion of the phosphorylated form slightly higher (Fig. 5). And resveratrol could return the changes to the level of untreated control. Resveratrol treatment did not affect the levels of its mRNA expression after UVA or TPA treatment (Fig. 6).

DISCUSSION

Sunlight coupled with living in oxygen-rich atmosphere causes unwanted and deleterious stresses on skin. The most severe consequence of photodamage is skin cancer. Less severe photoaging changes result in wrinkling, scaling, dryness, and mottled pigment abnormalities consisting of hyperpigmentation and hypopigmentation (Berneburg *et al.*, 2000; Yaar and Gilchrest, 2001; Kang *et al.*, 2001). Aged epidermis develops an abnormality in homeostasis, which is accentuated further in photoaged skin. Therefore, the homeostasis in epidermis is

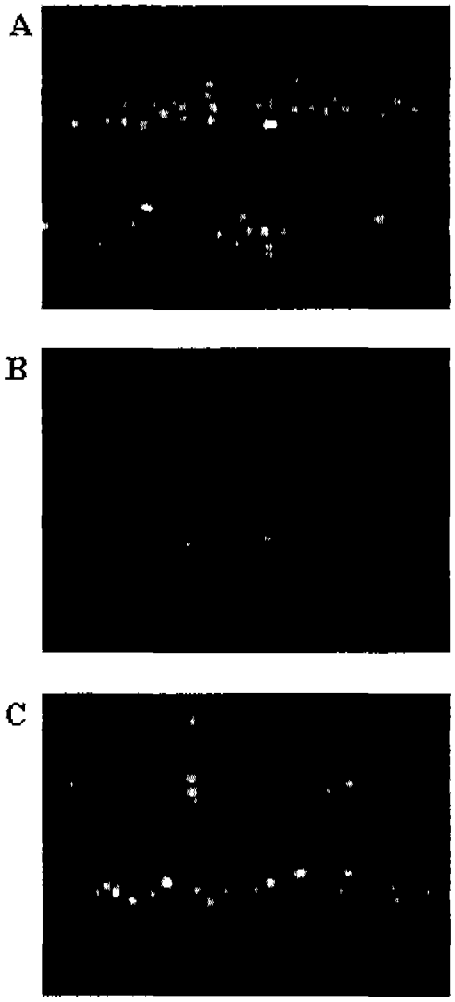

Fig. 3. Scrape loading/dye transfer (SL/DT) images in the HaCaT keratinocytes treated with resveratrol against TPA. Panel A, non-treated; Panel B, TPA 10 ng/ml; Panel C, TPA 10 ng/ml + resveratrol 1 μM.

very important to preserve the integrity of skin.

The intercellular signaling system mediated by connexin channels is one of crucial for maintaining tissue homeostasis, growth control, development, and synchronized response of cells to stimuli. Gap junctions are composed of a six transmembrane protein unit called a connexon (Kang *et al.*, 2001). Gap junction channels connect neighboring cells and allow intercellular transfer of ion as well as polarized and non-polarized molecules up to a molecular mass of 1 kDa (Kumar and Gilula, 1996). The most important permeates involved in cell signaling are probably cyclic nucleotide, inositol triphosphate, cAMP, calcium and other ions. Connexin 43 (Cx 43) is the most abundant connexin of human interfollicular epidermis, expressed throughout spinous and granular cell layers but also focally in

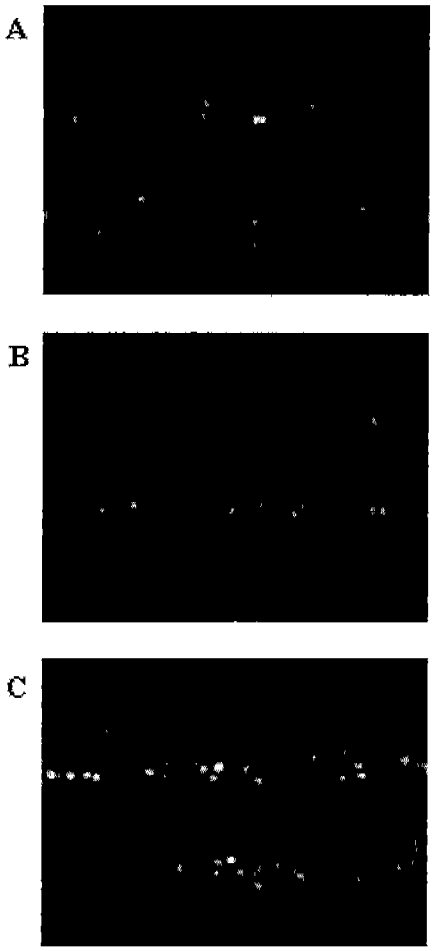

Fig. 4. Scrape loading/dye transfer (SL/DT) images in the HaCaT keratinocytes treated with resveratrol against UVA. Panel A, non-treated; Panel B, UVA 10 J/cm^2; Panel C, UVA 10 J/cm^2 + resveratrol 1 μM.

the basal cell layer (Guo *et al.*, 1992).

GJIC occurs in the epidermal and dermal compartments of normal fully differentiated human skin (Salomon *et al.*, 1988). The multiple co-expression of connexins by the same keratinocyte and their co-localization in the same GJ plaque suggests that the epidermis can form communication compartments with different regulatory properties (Risek *et al.*, 1998). The inhibition of GJIC between adjacent cells has been postulated to be one of the important events that take place during the promotional stage of cancer (Fitzgerald and Yamasaki, 1990; Yotti *et al.*, 1979). Besides of homeostasis, altered the regulation or expression of connexin protein is related to psoriasis, wound healing, cytokine regulation (Labarthe *et al.*, 1998; Abdullah *et al.*, 1999; Temme *et al.*, 1998). Recently, Statuto *et al.* (2002) have suggested that cell-cell communication through gap junctions is impaired in cell senescence.

DPPH (α,α-diphenyl-β-picrylhydrazyl) is a stable free radical with red color. If free radicals have been scavenged, DPPH will generate its color to yellow. This assay uses this character to evaluate free radical scavenging activity of the material. In the present study, resveratrol scavenged the DPPH radical like trolox, a well-known antioxidant. In DCF oxidation assay, UVA, UVB and TPA increased intracellular ROS productions and this increase was attenuated by treatment with resveratrol. Our results suggest that resveratrol has an antixoxidant activity and decreases both UV irradiation- and TPA-induced ROS productions. Ultraviolet irradiation like UVA and UVB can suppress the immune function of skin and promote skin cancer formation. The role of UVA in solar carcinogenesis is now gen-

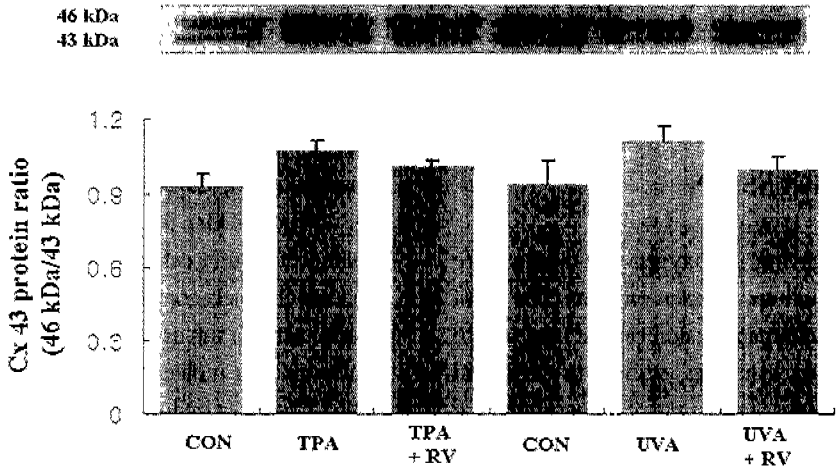

Fig. 5. Effects of resveratrol on Cx 43 protein expression in the HaCaT keratinocytes by Western blot analysis. CON, non-treated; TPA, TPA 10 ng/ml; TPA + RV, TPA 10 ng/μl + resveratrol 1 μM; CON, non-treated; UVA, UVA 10 J/cm^2; UVA + RV, UVA 10 J/cm^2 + resveratrol 1 μM.

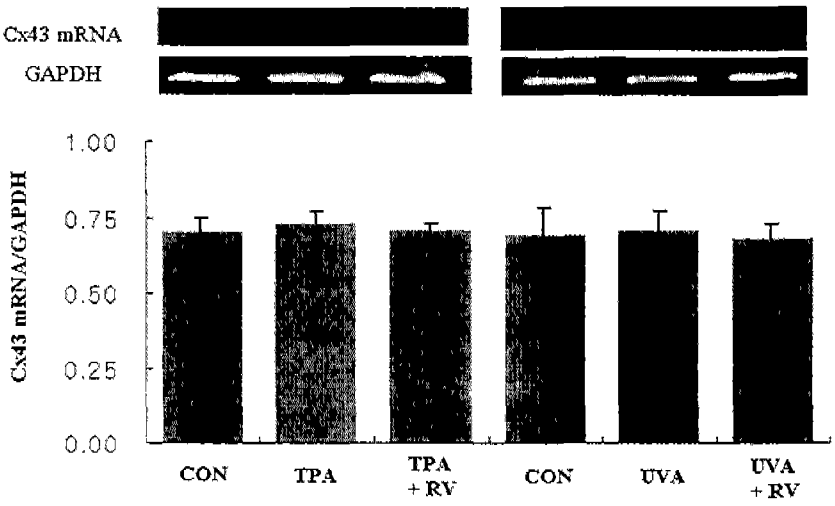

Fig. 6. Effects of resveratrol on Cx 43 mRNA level in the HaCaT keratinocytes. CON, non-treated; TPA, TPA 10 ng/ml; TPA + RV, TPA 10 ng/μl + resveratrol 1 μM; CON, non-treated; UVA, UVA 10 J/cm^2; UVA + RV, UVA 10 J/cm^2 + resveratrol 1 μM.

erally accepted (Zhang *et al.*, 2001). UVA irradiation displays a transient decrease in GJIC, which corresponds to Cx 43 internalization form plaque, as sometimes observed in tumor promotion (Holder *et al.*, 1993). The GJIC in HaCaT keratinocytes was inhibited by oxidative stress inducers such as ultraviolet A/B and TPA, possibly by activation of PKC. It has been reported that resveratrol inhibits several TPA-induced biomarkers, such as protein kinase C (PKC) activity (Stewart *et al.*, 1999), cyclooxygenase-2 (COX-2) expression in mammary and oral epithelial cells (Subbaramaiah *et al.*, 1998), formation of free radicals in HL-60 cells and cell transformation in the mouse JB6 epidermal cell line (Huang *et al.*, 1999). Resveratrol has the regulatory ability to the inhibition of GJIC on WB-F344 rat liver epithelial cells and inhibits tumorigenesis in mouse skin through interference with pathways of reactive oxidants and possibly by modulating the expression of c-*fos* and TGF-β1 (Nielsen *et al.*, 2000). Resveratrol has been also shown to suppress NF-κB activation induced by diverse oxidative or inflammatory stimuli, such as H_2O_2, oxidized lipoproteins, TNF-α, phorbol ester, and bacterial lipopolysaccharide (Draczynska-Lusiak *et al.*, 1998; Jang and Surh, 2001; Manna *et al.*, 2000). Chemopreventive potential of resveratrol is caused by antioxidant effects which can restore increased H_2O_2 levels and decreased glutathione levels to control levels induced by TPA treatment (Jang and Pezzuto, 1998). Similarly to TPA, H_2O_2 also induced the inhibition of GJIC in WB-F344 rat liver epithelial cell (Kang *et al.*, 2000). And H_2O_2-inhibited GJIC is restored after the removal H_2O_2 in WB liver epithelial cells (Upham *et al.*, 1997). Therefore, the preventive effects of resveratrol on GJIC inhibition by TPA or UVA might be involved in scavenging of the intracellular ROS such as H_2O_2. However, other mechanism can not be excluded.

Several researchers have suggested that the phosphorylation state of gap junction proteins seems to plays an important role in the gating of gap junction channels (Oh *et al.*, 1988; Rivedial *et al.*, 1994; Trosko and Ruch, 1998). Oxidative stresses such as ultraviolet and TPA induce the hyperphosphorylation of Cx 43. Uncontrolled phosphorylation of Cx 43 was reported to internalize into the cytoplasm, resulting in the loss of GJIC (Provost *et al.*, 2003). Hyperphosphorylation of Cx 43 is due to activation of PKC induced by TPA and UV irradiation in keratinocytes. In concert with hyperphosphorylation by PKC, Cx 43 was reported to internalize into the cytoplasm, resulting in the loss of GJIC. However in our study, we could not find significant changes of phosphorylation of Cx 43 and Cx 43 mRNA expression in our experimental conditions. We suggest that the reason why does not have significant changes is the time point difference between activation of PKC induced by TPA or UV irradiation and inhibition of GJIC. To explain this possibility, the study have to accomplished TPA and UV induced PKC activity and phosphorylation of Cx 43 in a time-dependent manner.

Our findings suggest that resveratrol has the protective effect on the oxidative stress-induced inhibition of GJIC of HaCaT keratinocyte, and this protection is likely due to the scavenging of reactive oxygen species. And this may be an important mechanism by which resveratrol preserves the homeostasis of skin and can prevent the oxidative stress-induced skin aging.

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