

Fucodiphlorethol G Purified from *Ecklonia cava* Suppresses Ultraviolet B Radiation-Induced Oxidative Stress and Cellular Damage

Ki Cheon Kim¹, Mei Jing Piao¹, Jian Zheng¹, Cheng Wen Yao¹, Ji Won Cha¹, Madduma Hewage Susara Ruwan Kumara¹, Xia Han¹, Hee Kyoung Kang¹, Nam Ho Lee² and Jin Won Hyun^{1,*}

¹School of Medicine and Institute for Nuclear Science and Technology,

²Department of Chemistry, College of Natural Sciences, Jeju National University, Jeju 690-756, Republic of Korea

Abstract

Fucodiphlorethol G (6'-[2,4-dihydroxy-6-(2,4,6-trihydroxyphenoxy)phenoxy]biphenyl-2,2',4,4',6-pentol) is a compound purified from *Ecklonia cava*, a brown alga that is widely distributed offshore of Jeju Island. This study investigated the protective effects of fucodiphlorethol G against oxidative damage-mediated apoptosis induced by ultraviolet B (UVB) irradiation. Fucodiphlorethol G attenuated the generation of 2, 2-diphenyl-1-picrylhydrazyl radicals and intracellular reactive oxygen species in response to UVB irradiation. Fucodiphlorethol G suppressed the inhibition of human keratinocyte growth by UVB irradiation. Additionally, the wavelength of light absorbed by fucodiphlorethol G was close to the UVB spectrum. Fucodiphlorethol G reduced UVB radiation-induced 8-isoprostane generation and DNA fragmentation in human keratinocytes. Moreover, fucodiphlorethol G reduced UVB radiation-induced loss of mitochondrial membrane potential, generation of apoptotic cells, and active caspase-9 expression. Taken together, fucodiphlorethol G protected human keratinocytes against UVB radiation-induced cell damage and apoptosis by absorbing UVB radiation and scavenging reactive oxygen species.

Key Words: Fucodiphlorethol G, Ultraviolet B, Mitochondria membrane potential, Reactive oxygen species, Human keratinocytes

INTRODUCTION

The ozone layer in the stratosphere has been progressively depleted since the late 1970s, leading to an excess amount of ultraviolet (UV) B irradiation reaching the earth's surface (Bollaji and Huan, 2013). Exposure to UVB radiation is the key factor in the initiation of several skin disorders, such as wrinkling, scaling, dryness, mottled pigment abnormalities including hypo/hyperpigmentation, and skin cancer (Drouin and Therrien, 1997). UVB radiation may lead to apoptosis *via* directly and indirectly causing DNA damage (Ravanat *et al.*, 2001), although it may also lead to production of vitamin D, which is essential for the prevention of osteoporosis *via* calcium metabolism in skin cells (Holick, 2004). UVB radiation can modify molecular structures and damage cellular components, and thereby disrupt cellular functions (Petrova *et al.*, 2011; Wölfle *et al.*, 2011). UVA irradiated from sunlight can contribute to skin cancer *via* indirect DNA damage induced by reactive oxygen

species (ROS) and free radicals. While the damage caused by UVB induced both indirect DNA damage like as ROS generation and direct DNA damage such as formation of pyrimidine dimers, and double-strand DNA breakage (Svobodová *et al.*, 2012). UVB has higher energy per photon than UVA, but most UVB radiation was blocked by ozone layer. However, the rate of UVB reached to earth surface was increased by ozone depletion induced by green-house gases. While the increasing of UVB radiation exposure to human being, the researches of UVB protection have been important. As the risk of UVB radiation causing skin damage, such as inflammation, erythema, and skin carcinomas, has increased, investigations have sought to identify natural extracts and compounds that can block UVB radiation and protect against its damaging effects. Brown alga polyphenols have an anti-photocarcinogenic effect, which may be associated with the prevention of UVB radiation-induced oxidative stress, inflammation, and cell proliferation in the skin (Hwang *et al.*, 2006). For example,

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*Corresponding Author

E-mail: jinwonh@jejunu.ac.kr

Tel: +82-64-754-3838, Fax: +82-64-702-2687

alga extract from *Phaeodactylum tricornutum* stimulates the activity of proteasome peptidase, which is responsible for the degradation of most oxidized proteins, in human keratinocytes exposed to UVB irradiation (Bulteau *et al.*, 2006). A *Chlorella* extract prevents UVB radiation-induced matrix metalloproteinase 1 expression and UVB radiation-suppressed elastin protein expression and pro-collagen gene expression in skin fibroblasts (Shih and Cherng, 2008). Our recent studies reported that eckol and triphlorethol-A compounds purified from *Ecklonia cava* protect against UVB radiation-induced cell damage and apoptosis in human keratinocytes (Piao *et al.*, 2012a, 2012b).

Fucodiphlorethol G (6'-[2,4-dihydroxy-6-(2,4,6-trihydroxyphenoxy)phenoxy]biphenyl-2,2',4,4',6-pentol), a phlorotannin component of *Ecklonia cava*, scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Ham *et al.*, 2007). Although the effects of radical scavenging by fucodiphlorethol G have been studied, the cytoprotective effect of this compound against UVB exposure has not been investigated. The current study investigated the protective properties of fucodiphlorethol G against UVB radiation-induced damage and apoptosis in human keratinocytes.

MATERIALS AND METHODS

Reagents

Fucodiphlorethol G was provided by Professor Nam Ho Lee (Jeju National University, Jeju, Korea) (Fig. 1). DPPH, 2,7-dichlorodihydrofluorescein diacetate (DCF-DA), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT), Hoechst 33342, and an anti-actin antibody were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). A caspase-9 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and UVB irradiation

Human keratinocytes (HaCaT cells), obtained from Amore Pacific Company (Yongin, Korea), were maintained at 37°C in an incubator in a humidified atmosphere of 5% CO₂. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml), and penicillin (100 U/ml). The UVB source was a CL-1000M UV Crosslinker (UVP, Upland, CA, USA), which was used to deliver an energy spectrum of UVB radiation (280-320 nm; peak intensity, 302 nm).

DPPH radical detection

Fucodiphlorethol G (1, 10, 20, 40, 60, 80, or 100 µM) and 1 mM N-acetylcysteine (NAC) was added to 0.1 mM DPPH and mixed well. The mixture was incubated for 30 min, after which the amount of residual DPPH was determined by measuring absorbance at 520 nm using a spectrophotometer.

Intracellular reactive oxygen species (ROS) detection

The DCF-DA method was used to detect intracellular ROS levels in HaCaT keratinocytes (Rosenkranz *et al.*, 1992). H₂O₂-treated or UVB-irradiated cells were seeded at a density of 0.5×10⁵ cells/well in 24-well culture plates. Sixteen hours after plating, cells were treated with fucodiphlorethol G at a concentration of 1, 10, 20, 40, 60, 80, or 100 µM or with 1 mM NAC. To analyze 2,7-dichlorofluorescein (DCF) gener-

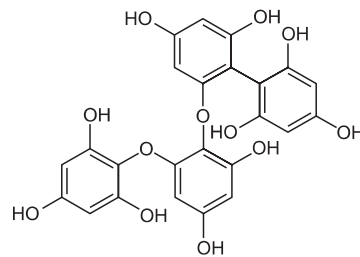


Fig. 1. Chemical structure of fucodiphlorethol G (6'-[2,4-dihydroxy-6-(2,4,6-trihydroxyphenoxy)phenoxy]biphenyl-2,2',4,4',6-pentol).

ated by intracellular ROS, cells were treated with 1 mM H₂O₂ or irradiated with 30 mJ/cm² UVB. After incubating for 30 min or 12 h, 25 µM DCF-DA was added and cells were incubated for a further 10 min. Fluorescence of DCF was detected using a PerkinElmer LS-5B spectrofluorometer (PerkinElmer, Waltham, MA, USA).

Cell viability

Cells were seeded in a 24-well plate at a density of 0.5×10⁵ cells/well. Sixteen hours after plating, cells were treated with fucodiphlorethol G at a concentration of 1, 10, 20, 40, 60, 80, or 100 µM or with 1 mM NAC. Cells were exposed to 30 mJ/cm² UVB radiation 1 h later and incubated at 37°C for 48 h. Thereafter, 150 µl of MTT stock solution (2 mg/ml) was added to each well to yield a total reaction volume of 500 µl. After incubating cells for 2 h, the plate was centrifuged at 800×g for 5 min, and the supernatants were aspirated. The formazan crystals in each well were dissolved in dimethylsulfoxide (DMSO), and the absorbance at 540 nm was measuring using a scanning multi-well spectrophotometer (Carmichael *et al.*, 1987).

UV/visible light absorption analysis

An absorption analysis of fucodiphlorethol G (20 µM) was performed by scanning with UV/visible light of 200-400 nm using an HP-8453E UV-visible spectroscopy system (Hewlett Packard, Palo Alto, CA, USA) and a standard quartz cuvette with a 1 cm path length. Fucodiphlorethol G was diluted in DMSO at a ratio of 1:500 before scanning.

Analysis of the level of 8-isoprostane

Lipid peroxidation was assayed by determining the level of 8-isoprostane in culture medium (Belli *et al.*, 2005). Cells were treated with 20 µM fucodiphlorethol G, exposed to UVB radiation 1 h later, and incubated for an additional 48 h at 37°C. An enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) was used to measure the level of 8-isoprostane according to the manufacturer's protocol.

DNA fragmentation analysis

Cells were seeded in a 24-well plate at a density of 0.5×10⁵ cells/well. At sixteen hours after plating, cells were treated with 20 µM fucodiphlorethol G, exposed to UVB radiation 1 h later, and incubated for an additional 48 h at 37°C. Cellular DNA fragmentation was assessed by analyzing cytoplasmic histone-associated DNA fragmentation. A kit from Roche Diagnostics (Portland, OR, USA) was used according to the manufacturer's protocol.

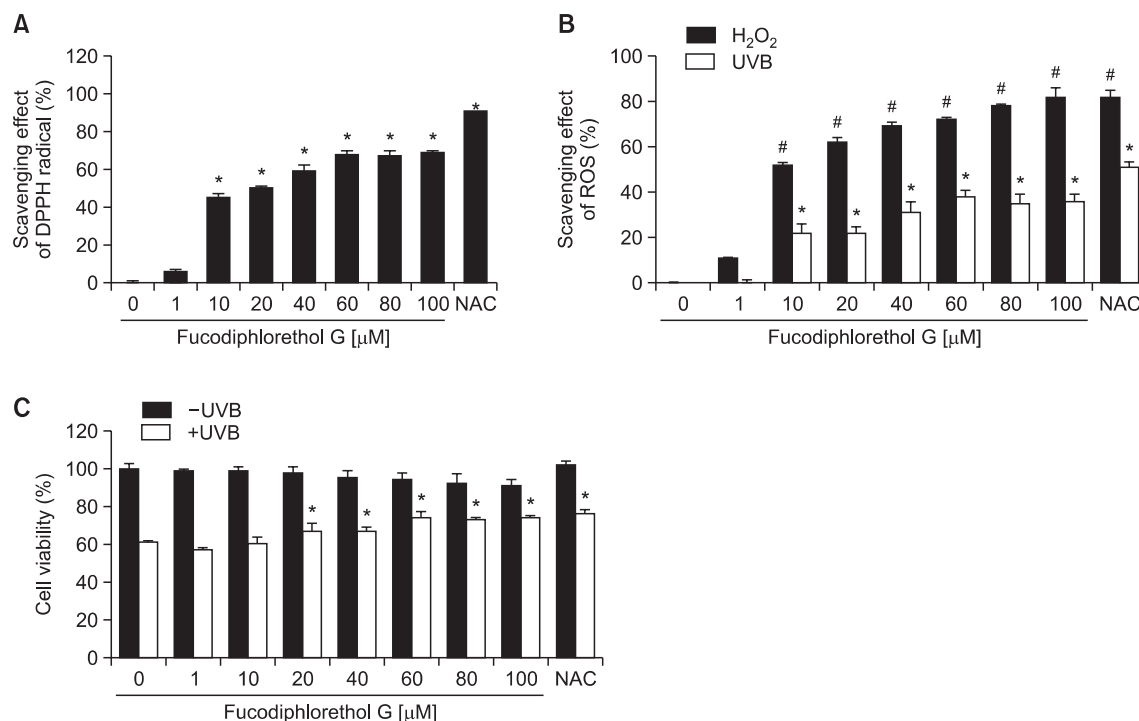


Fig. 2. Fucodiphlorethol G scavenges DPPH radical and intracellular ROS and protects against cell death induced by UVB. (A) DPPH radical was incubated with various concentrations of fucodiphlorethol G. The amount of DPPH radical was determined by measuring absorbance at 520 nm using a spectrophotometer. *Significantly different from control ($p < 0.05$). (B) Cells were treated with various concentrations of fucodiphlorethol G for 1 h and then treated with 1 mM H₂O₂ or 30 mJ/cm² UVB radiation. Cells were incubated for a further 24 h and intracellular ROS were detected by fluorescence spectrophotometry. #*Significantly different from each control in H₂O₂ or UVB-treated cells, respectively ($p < 0.05$). (C) Cells were treated with various concentrations of fucodiphlorethol G for 1 h and then treated with 30 mJ/cm² UVB radiation. Cell viability was determined using the MTT assay. *Significantly different from UVB-irradiated cells ($p < 0.05$).

Mitochondrial membrane potential ($\Delta\psi_m$) analysis

Cells were treated with fucodiphlorethol G (20 μ M), exposed to UVB radiation (30 mJ/cm²) 1 h later, and then incubated for another 12 h at 37°C. Cells were stained with JC-1 (15 μ M). For image analysis, JC-1-stained cells were mounted in mounting medium (DAKO, Carpinteria, CA, USA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope. In addition, JC-1-stained cells were analyzed by flow cytometry (Troiano *et al.*, 2007).

Western blot analysis

Cell lysates were collected and protein concentrations were determined using the Bradford reagent. Aliquots of the lysates (20 μ g of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Gels were transferred to nitrocellulose membranes (Bio-Rad, CA, USA). Membranes were incubated with a caspase-9 antibody and further incubated with secondary immunoglobulin G-horseradish peroxidase conjugates. Protein bands were visualized by developing the blots using an Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham, Buckinghamshire, UK) and exposing the membranes to X-ray film.

Nuclear staining with Hoechst 33342

Cells were treated with 20 μ M fucodiphlorethol G and exposed to 30 mJ/cm² UVB radiation 1 h later. After incubation for a further 48 h at 37°C, 1 μ l of the DNA-specific fluorescent dye

Hoechst 33342 (stock, 15 mM) was added to each well of the 6-well plate. The plate was then incubated for 10 min at 37°C. The degree of nuclear condensation in the stained cells was determined by visualization with a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera.

Statistical analysis

All measurements were made in three independent experiments, and all values represent the mean \pm standard error. The results were subjected to an analysis of variance using Tukey's test for analysis of differences. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of fucodiphlorethol G on free radical scavenging and on the viability in UVB-irradiated cells

Fucodiphlorethol G significantly scavenged DPPH radical in a concentration-dependent manner (Fig. 2A). The intracellular ROS scavenging effect of fucodiphlorethol G in H₂O₂ (1 mM)-treated cells or UVB (30 mJ/cm²)-irradiated cells increased in a concentration-dependent manner (Fig. 2B). Moreover, these antioxidant effects of fucodiphlorethol G increased cell viability reduced by UVB in a concentration-dependent manner; the survival rates of UVB-irradiated cells pre-treated with 20, 40, 60, 80, and 100 μ M fucodiphlorethol G were 67%, 67%, 74%, 73%, and 74%, respectively in comparison to that of cells only

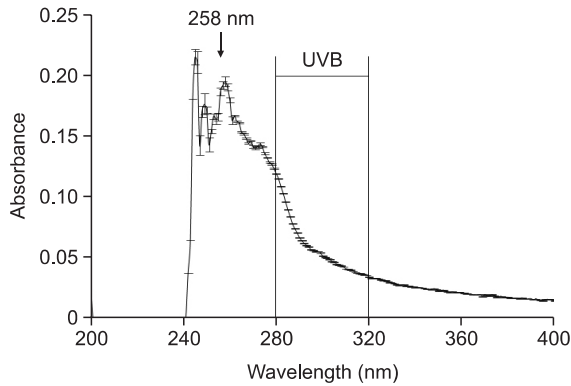


Fig. 3. Fucodiphloretol G absorbs UVB light. UV/visible spectroscopic measurements were conducted in the spectral range of 200-400 nm to measure absorbance by fucodiphloretol G.

exposed to UVB radiation (61%) (Fig. 2C), suggesting that ROS scavenging effect of fucodiphloretol G are related to protection of cells against oxidative stress induced by UVB radiation. Moreover, treatment with 20 μ M fucodiphloretol G was non-toxic to cells. Thus, we chose 20 μ M as the optimal concentration of fucodiphloretol G for subsequent experiments. These data suggest that fucodiphloretol G not only scavenges free radicals and intracellular ROS generated by UVB irradiation, but also protects cells from UVB radiation-induced cell death.

Effects of fucodiphloretol G on absorption of light within the UVB spectrum

UVB radiation has a wavelength of 280-320 nm. The effect of fucodiphloretol G on absorption of UVB was determined using a UV/visible spectrophotometer. Fucodiphloretol G showed a high absorptive capacity at a peak position of 258 nm, which is close to the range of UVB radiation (Fig. 3). Thus, the light-absorbing property of fucodiphloretol G might be closely associated with its cytoprotective effect against UVB radiation. This data show that fucodiphloretol G absorbed UVB radiation and may reduce the level of UVB radiation that reaches cells.

Protective effects of fucodiphloretol G against damage to cellular components caused by UVB radiation

To investigate the protective effects of fucodiphloretol G against membrane lipid peroxidation and cellular DNA damage induced by UVB irradiation, cells were treated with fucodiphloretol G, exposed to UVB 1 h later, and then incubated for a further 48 h. 8-Isoprostane is a by-product of ROS-induced lipid peroxidation and is used as a specific index of cellular iperoxidation (Belli *et al.*, 2005). The concentration of 8-isoprostane in UVB-treated cells was significantly increased to 91 pg/ml, whereas this was reduced to 60 pg/ml in UVB-irradiated cells pretreated with fucodiphloretol G (Fig. 4A). Moreover, while the level of DNA fragmentation was higher in UVB-irradiated cells than in control cells, it was significantly reduced in UVB-irradiated cells pretreated with fucodiphloretol G (Fig. 4B). These data indicate that fucodiphloretol G attenuated lipid peroxidation and DNA fragmentation induced by UVB irradiation in HaCaT cells.

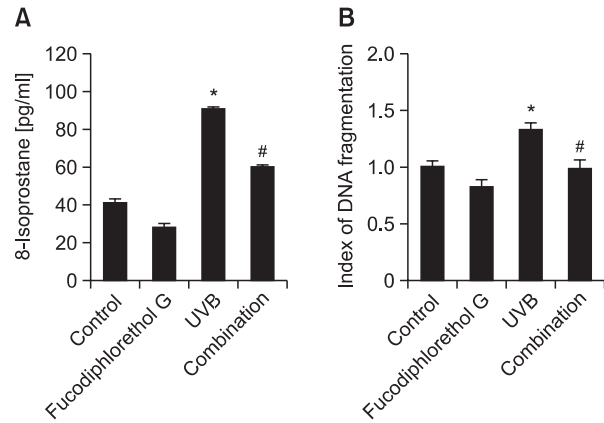


Fig. 4. Fucodiphloretol G protects HaCaT keratinocytes against UVB radiation-induced lipid peroxidation and DNA fragmentation. (A) The level of 8-isoprostane, an indication of lipid peroxidation, was measured in culture medium. *Significantly different from control cells ($p < 0.05$); #significantly different from UVB-irradiated cells ($p < 0.05$) (B) DNA fragmentation was quantified with an enzyme-linked immunosorbent assay kit. *Significantly different from control cells ($p < 0.05$); #significantly different from UVB-irradiated cells ($p < 0.05$).

Effects of fucodiphloretol G on UVB radiation-induced disruption of $\Delta\psi_m$ and apoptosis

Mitochondria are the site at which oxidative phosphorylation occurs and are intimately involved in regulation of apoptosis, ROS production during respiration, and regulation of cellular metabolism. To understand the mechanism by which fucodiphloretol G protects against UVB radiation-induced apoptosis, $\Delta\psi_m$ was examined. Strong red fluorescence, indicative of JC-1 aggregates and mitochondrial polarization, was exhibited in control cells and fucodiphloretol G-treated cells (Fig. 5A). However, in cells exposed to UVB radiation, the level of red fluorescence was reduced and green fluorescence was observed, indicative of the JC-1 monomer and mitochondrial depolarization. Pretreatment with fucodiphloretol G prevented both the reduction in red fluorescence and the induction of green fluorescence in UVB-irradiated cells. To confirm this data, it was investigated the levels of green fluorescence of JC-1, indicative of a loss of $\Delta\psi_m$, using flow cytometry (Fig. 5B). UVB-irradiated cells showed a loss of $\Delta\psi_m$, as evidenced by an increase in green fluorescence, whereas pre-treatment with fucodiphloretol G suppressed the loss of $\Delta\psi_m$ in UVB-irradiated cells. Disruption of $\Delta\psi_m$ causes early apoptosis *via* production of the apoptosome. The level of active caspase-9, one of the components of the apoptosome, was measured by western blotting. Treatment with fucodiphloretol G markedly attenuated the level of active caspase-9 in comparison to that in cells only exposed to UVB radiation (Fig. 5C). To further study the protective effect of fucodiphloretol G against UVB radiation-induced apoptosis in HaCaT cells, nuclei were stained with Hoechst 33342 and visualized by fluorescence microscopy. Control cells showed intact nuclei, whereas large amounts of nuclear shrinkage and fragmentation were observed in UVB-irradiated cells. Although UVB irradiation caused apoptotic nuclei in HaCaT cells, pre-treatment with fucodiphloretol G significantly reduced the amounts of nuclear shrinkage and fragmentation (Fig. 5D). Taken together, these

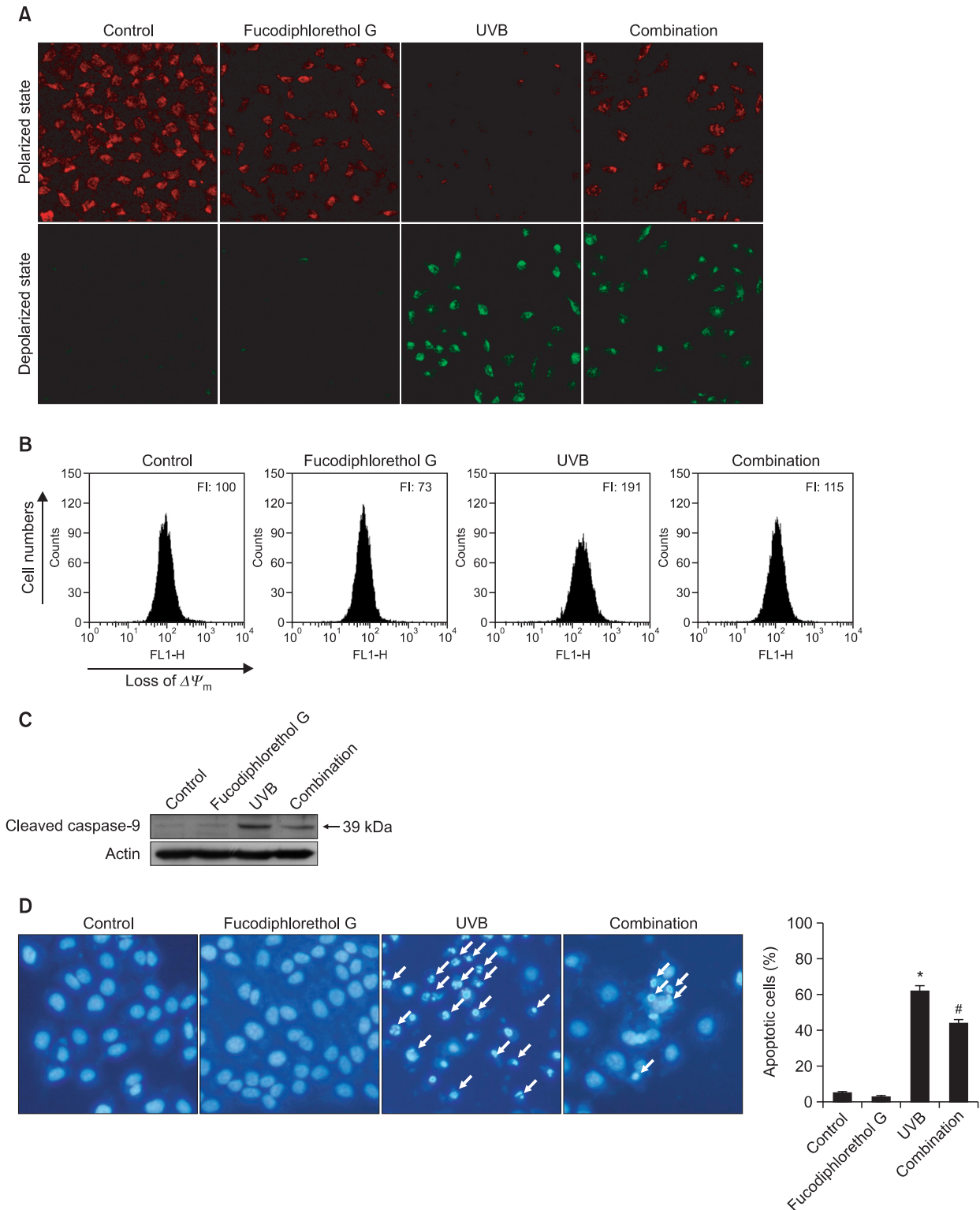


Fig. 5. Fucodiphloretol G suppresses UVB radiation-induced disruption of $\Delta\Psi_m$, activation of caspase-9, and apoptosis in HaCaT keratinocytes. Cells were pre-treated with 20 μ M fucodiphloretol G and then treated with 30 mJ/cm^2 UVB radiation 1 h later. Cells were stained with JC-1, and $\Delta\Psi_m$ was analyzed by (A) confocal microscopy and (B) flow cytometry. (C) Cleaved caspase-9 was detected using a specific antibody. (D) Apoptotic body formation was observed under a fluorescence microscope following Hoechst 33342 staining. Apoptotic bodies are indicated by arrows. *Significantly different from control cells ($p < 0.05$); #Significantly different from UVB-irradiated cells ($p < 0.05$).

data indicate that treatment with fucodiphloretol G protected HaCaT cells against UVB radiation-induced apoptotic cell death.

DISCUSSION

Abnormal weather conditions have become a serious problem, along with global warming caused by the accumulation of greenhouse gases (McKenzie *et al.*, 2011). These conditions exacerbate degradation of the ozone layer, leading to exposure of the earth's surface to sunlight containing a large amount of UVB radiation (Bais *et al.*, 1993; McKenzie *et al.*, 2011). Although UVB radiation has beneficial effects, such as vitamin D synthesis, on organisms, exposure to excessive amounts of UVB radiation can cause cellular damage and cell death (Drouin and Therrien, 1997; Ali *et al.*, 2011; Wacker and Holick, 2013). In particular, upon exposure to excessive amounts of UVB radiation, skin cells can undergo death and skin cancer often develops (Drouin and Therrien, 1997; Ali *et al.*, 2011). These effects of UVB radiation are due to the induction of DNA damage and oxidative stress *via* ROS generation in skin cells (Ravanat *et al.*, 2001; Fagot *et al.*, 2002; Ichihashi *et al.*, 2003). In the current study, we focused on UVB radiation-induced ROS and the protective effects of fucodiphloretol G against oxidative stress. DPPH radical and intracellular ROS induced by treatment with hydrogen peroxide or UVB irradiation were scavenged by fucodiphloretol G in a concentration-dependent manner (Fig. 2). Additionally, fucodiphloretol G restored the UVB radiation-induced reduction in cell viability. In addition to its ROS-scavenging effects, fucodiphloretol G also directly absorbed UVB radiation (Fig. 3). Oxidative stress induced by UVB radiation modifies various cellular components such as lipid membranes, proteins, DNA, and mitochondria. These modifications lead to perturbation of the functions of these structures, after which repair or apoptotic mechanisms are initiated (Kulms and Schwarz, 2002; Sander *et al.*, 2002; Caricchio *et al.*, 2003). UVB-irradiated cells exhibited increased lipid peroxidation and DNA fragmentation (Fig. 4). Meanwhile, fucodiphloretol G pretreatment suppressed UVB radiation-induced lipid peroxidation and DNA fragmentation. Furthermore, $\Delta\psi_m$ was disrupted upon exposure to UVB irradiation and restored by pre-treatment with fucodiphloretol G. A decreased $\Delta\psi_m$ should induce release of cytochrome c from mitochondria, which is a critical component of the intrinsic apoptotic pathway (Arends *et al.*, 1990; Tsujimoto, 1998). The release of cytochrome c from mitochondria into the cytosol triggers formation of the apoptosome, which is composed of apoptotic protease activating factor 1, cytochrome c, and active caspase-9. Fucodiphloretol G suppressed UVB radiation-induced active caspase-9 and apoptotic cell death (Fig. 5).

In summary, UVB radiation induces oxidative stress in human keratinocytes and eventually damages cells and causes apoptosis. However, fucodiphloretol G blocks UVB radiation-induced oxidative stress and cell damage *via* reducing the level of ROS, directly absorbing UVB radiation, and blocking mitochondrial dysfunction, resulting in the cytoprotection against UVB radiation-induced apoptosis.

ACKNOWLEDGMENTS

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