

Protective effects of ginsenoside Rg2 and astaxanthin mixture against UVB-induced DNA damage

Yu Heon Chung^a, Seul A. Jeong^a, Hyun Seok Choi^a, Seungil Ro^b, Jung Sup Lee^c and Jong Kun Park^a

^aResearch Institute for Basic Science and Division of Biological Science, Wonkwang University, Iksan, Korea; ^bDepartment of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, NV, USA; ^cDepartment of Biomedical Science, BK21-plus Research Team for Bioactive Control Technology, College of Natural Sciences, Chosun University, Gwangju, Republic of Korea

ABSTRACT

Ultraviolet B (UVB) radiation induces skin damage, skin matrix degradation, and wrinkle formation through photochemical reaction and oxidative stress. Therefore, protecting the skin from UVB can prevent skin aging. In this study, we investigated the effects of a mixture (RA) of Rg2, a ginsenoside, and astaxanthin, an antioxidant, on the responses of HaCaT cells exposed to UVB (700 J/m²). The cells were incubated for 24 h after UVB exposure and cell viability was determined by MTT assay. UVB decreased cell viability by 60% compared to that of untreated control cells, whereas RA increased cell viability in a concentration-dependent manner, and this increase was significantly higher than that in the single treatment groups. Further, UVB increased the levels of DNA lesions such as cyclobutane pyrimidine dimer (CPD) and 8-hydroxyguanine (8-OHdG). Conversely, RA decreased both CPD and 8-OHdG levels in a concentration-dependent manner. UVB exposure also increased phosphorylation of ataxia-telangiectasia mutated (ATM) protein kinase and p53 and subsequently increased the levels of GADD45 α , p21, and matrix metalloproteinases (MMPs)-3, -9, and -13. Additionally, UVB exposure decreased the level of COL1A1. However, RA treatment decreased the levels of p-ATM, p-p53, GADD45 α , p21, MMP-3, -9, and -13 and increased the level of COL1A1 in a concentration-dependent manner. These results suggest that RA reduces UVB-induced cytotoxicity and genotoxicity through up-regulation of DNA repair via the combined effects of Rg2 and astaxanthin.

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Introduction

Solar radiation (UV) has been well-known for its genotoxicity and strong mutagenic effects. UV irradiation usually results in cellular death, genomic instability, and mutations in oncogenes and tumor-suppressor genes. These mutations can lead to abnormal cell growth, increasing the risk of cancer development. Solar ultraviolet radiation (UV) is classified as UVA (320–400 nm), UVB (280–320 nm), and UVC (280–100 nm) according to their wavelengths. The short-wavelength UVC is absorbed by the ozone layer and does not reach the Earth's surface. However, UVA and UVB can reach the Earth's surface and affect terrestrial organisms (Mastumura and Ananthaswamy 2004; Kozma and Eide 2014). Among the two, UVB is the more potent genotoxic agent; exposure to UVB radiation both burns the skin and drives the initiation, promotion, and progression of skin carcinogenesis. In particular, absorption of UVB radiation directly induces DNA damage through the formation of DNA lesions, such as cyclobutane pyrimidine

dimer (CPD) and 8-hydroxyguanine (8-OHdG) (Lo et al. 2005; Aitken et al. 2007). These lesions have been shown to obstruct base pairing and interrupt DNA replication and transcription. These types of DNA damage induce mutation of the p53 tumor suppressor gene, and thus, are crucial for cell proliferation and apoptosis and enhance the risk of photocarcinogenesis. The UVB-mediated damaged cells respond to these alterations by either activating their DNA repair mechanism or inducing apoptotic death when the damage is extreme.

The tumor suppressor protein, p53, is an important transcription factor that is induced in response to various cellular stresses including UV light, ionizing radiation, chemical carcinogens, and chemotherapeutic agents (Liu and Kulesz-Martin 2001; Waster and Ollinger 2009). Nucleotide excision repair (NER) is one of the major types of DNA repair pathways responsible for removal of bulky UV-induced DNA lesions, such as 6–4 photoproducts and CPD. Regulation of NER is mediated by p53 (Fischer et al. 2007). When p53 is activated, it

CONTACT Jong Kun Park  jkpark@wku.ac.kr  Research Institute for basic Science and Division of Biological Science, Wonkwang University, Iksan 570-749, Korea

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regulates its downstream pathways, including DNA repair, cell cycle arrest (senescence), or apoptosis in response to DNA damage, by transcriptionally regulating growth arrest and the DNA damage-inducible protein GADD45 α , cyclin-dependent kinase (CDK) inhibitor p21, and proapoptotic BAX and PUMA proteins (Waster and Ollinger 2009; Ciccia and Elledge 2010). As a p53 target, GADD45 α regulates various processes involving G2 cell cycle arrest, apoptosis, DNA repair, and genomic stability in response to DNA damage (Fayolle et al. 2008; Liebermann and Hoffman 2008). The p53-dependent p21 is a mediator of G1 cell cycle arrest following DNA damage (Laskin and Pendino 1995; Xue et al. 2007). Thus, p53 is a DNA-binding transcription factor that plays an important role in regulating DNA damage response.

Ginseng, the root of *Panax ginseng*, is a well-known traditional medicine that contains ginsenosides as its major active ingredients (Chang et al. 2008). Ginsenosides include Rg1, Rg2, Rb2, and Rh1, among which Rg2, a triterpenoid saponin, has been reported to exhibit anti-oxidant, anti-diabetic, anti-apoptotic and neuroprotective activities (Yun and Choi 1995; Jeong et al. 2007; Zhang et al. 2008; Ye et al. 2016). Rg2 inhibited lipopolysaccharide (LPS)-induced VCAM-1 and ICAM-1, prevented I κ B degradation, and blocked leukocyte attachment in an atherosclerosis model. Moreover, Rg2 decreased LPS-induced BAX, and caspase-3 and -9 expression and exhibited an anti-apoptotic activity in neuron cells. Besides, Rg2 decreased phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase levels (Ye et al. 2016). Rg2 has also been reported to inhibit glucogenesis through phosphorylation of AMP-activated protein kinase (AMPK) and glycogen synthase kinase 3 β (GSK-3 β) in liver cells (Yuan et al. 2012). Additionally, Rg2 was shown to protect PC12 cells against glutamate-induced neurotoxicity (Li et al. 2007). In an Alzheimer's disease (AD) mouse model, Rg2 increased the levels of dihydrosphingosine and lysophosphatidylcholine and decreased the hypoxanthine level (Li et al. 2016). Rg2 also has pharmacological activities, including improvement of neurological performance and memory, protection against UVB-induced DNA damage, and decreasing apoptosis (Jeong et al. 2007; Zhang et al. 2008; Ha et al. 2010).

Astaxanthin (ASTA), known as red carotenoid pigment, belongs to the xanthophylls class of carotenoids (Yang et al. 2011). ASTA is naturally found in a wide variety of living organisms, such as microalgae, salmonoids, fungi, and crustaceans. It has been reported to perform important metabolic functions in animals, including potent anti-tumoral, anti-oxidant, anti-inflammatory, neuroprotection, and protection against

diseases such as cancer. Besides, ASTA is known for its remarkable antioxidant effects (Yeyah et al. 2012); the antioxidant activity of ASTA has been reported to be more effective than those of other carotenoids, including lutein, canthaxanthin, and beta-carotene. These effects are considered a defense mechanism against attack by reactive oxygen species (Byeon et al. 2012). Moreover, ASTA has been shown to strongly inhibit oxygen radical-mediated lipid peroxidation, singlet oxygen formation by quenching its generation, and hydrogen peroxide-mediated apoptotic cell death (Yang et al. 2011; Byeon et al. 2012). Therefore, ASTA is a potent antioxidant agent.

In this study, we investigated the effects of a mixture (RA) of Rg2 and ASTA on the UVB-induced DNA damage responses in human keratinocyte HaCaT cells.

Methods

Reagents

Rg2 and astaxanthin were purchased from Sigma Aldrich (USA). Rg2 and astaxanthin were dissolved in dimethyl sulfoxide (DMSO) and PBS, respectively, and stored at -20°C .

Cell culture and UVB radiation

HaCaT cells were grown in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), in a humidified atmosphere with 5% CO₂ at 37°C. The cells were exposed to the indicated dose of UVB using a germicidal lamp (Sankyo Denki Co., Japan) in a UV box. The dose of UV-irradiation was measured using a UV radiometer (UVB Inc., USA). In this study, the cells were incubated 24 h before irradiation and washed once with PBS.

Measurement of cell viability

HaCaT cells were cultured in 96-well tissue culture plates (SPL, Korea) and maintained in 200 μL of RPMI 1640 supplemented with 10% FBS. After 24 h, the cells were washed once with PBS, exposed to UVB, and post-incubated with various concentrations of Rg2, astaxanthin, or the mixture of Rg2 and astaxanthin (RA). After 24 h, the cells were washed twice with PBS, treated with 150 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) solution (0.5 mg/mL in PBS) per well and incubated for an additional 4 h at 37°C. The MTT solution was then removed and the formed formazan was dissolved in 50 μL DMSO. The

conversion of MTT to formazan was quantified per well at 570 nm, using an ELISA reader (Retisoft Inc., Canada).

Immunodot blotting (IDB)

For IDB analysis, triplicate samples of 1 µg heat-denatured DNA per dot were loaded on a positively charged polyvinylidene fluoride membrane (PVDF, 0.45 µm) (Millipore, Germany). After blotting, the dots were rinsed twice with 100 µL TBS containing 0.05% Tween 20 (TBS-T) and incubated with peroxidase-conjugated anti-CPD (Cosmo Bio, Japan) and anti-8-OHdG (Santa Cruz Biotechnology, USA) antibodies (1:5000 dilution in 2% skim milk) at 37°C for 3 h. The blots were then washed with TBS-T, peroxidase activity was detected using the enhanced blotting detection system, and the membranes immediately exposed to X-ray films (Fuji, Japan) at different time points.

Western blotting

Cells were lysed with protein prep buffer (Intron, Korea) and boiled for 10 min. A 50 µg protein from each cell lysate was subjected to electrophoresis and separated on 10% SDS-polyacrylamide gel. The samples were then electroblotted onto a PVDF membrane. After blocking, the blots were incubated with p-ATM, ATM, p-p53, p53, GADD45α, p21^{Waf1/Cip1} (Cell Signaling, USA), MMP-3, -9, -13, COL1A1, and β-actin (Santacruz, USA) antibodies for 2 h. After washing twice with TBS-T for 15 min, the membranes were incubated with horseradish peroxidase-conjugated rabbit or goat anti-mouse IgG secondary antibody for 1 h. After washing, the membranes were treated with western blotting reagent ECL Plus (ELPIS, Korea) and immediately exposed to X-ray films at different time points. Densitometric measurements of band intensities for target proteins and β-actin, a loading control, were performed using Image J software (NIH, Bethesda, MA, USA).

Statistical analysis

All the data obtained in the present study were compared using the Student's *t*-test to determine the statistical significance between the groups. Throughout the text, values are expressed as the mean ± SD of at least three independent experiments.

Results and discussion

In the present study, we studied the effects of ginsenoside Rg2 and astaxanthin mixture on the DNA damage responses of UVB-exposed HaCaT cells. In the absence

of UVB exposure, individual Rg2 and ASTA treatments only slightly changed the viability of the cultured cells in growth medium (data not shown). When cells were exposed to 700 J/m² UVB, the cell viability was reduced to approximately 50% as compared to that of the untreated control cells (Figure 1). However, the post-incubation of UVB-exposed cells in medium containing either Rg2 or ASTA significantly increased the cell viability in a concentration-dependent manner compared to that in UVB-exposed untreated cells (Figure 1(A,B)). Then we checked the effect of different combinations of Rg2 and ASTA at various ratios to determine the most effective ratio against UVB-induced cytotoxicity and found that RA with 5:1 ratio yielded the highest cell viability (data not shown). The concentration-dependent cytoprotection offered by resulting RA was more than provided that by individual treatments of Rg2 or ASTA (Figure 1(C)).

To determine the rate of removal of CPD and 8-OHdG from the genomic DNA of HaCaT cells exposed to UVB, these photoproducts were measured by IDB (Figure 2(A,B)). Treatment with ASTA alone did not change the level of UVB-induced photoproducts compared to that in the untreated control cells (data not shown). In contrast, treatment with RA for 24 h post UVB exposure decreased the level of CPD and 8-OHdG in a concentration-dependent manner (Figure 2(A,B)). These results demonstrated the protective effects of RA against UVB-induced DNA damages.

p53 is known to be involved in DNA repair by at least two pathways: First, p53 transactivates XPC and XPE, both of which are involved in nucleotide excision repair (NER) and increase global genomic repair. Second, p53 is reported to increase DNA repair by direct protein interaction. For example, p53 activates OGG1 and APE, members of base excision repair (BER), and thus decreases levels of 8-OHdG, a marker of aging (Achanta and Huang 2004). Besides the abovementioned NER and BER, p53 is reported to be involved in other repair pathways, including mismatch repair, homologous recombination, and translesion synthesis (McCulloch et al. 2004; Romanova et al. 2004; Kunkel and Erie 2005).

To confirm the effect of RA on the expression levels of DNA damage marker proteins, we determined the expression of phospho-ATM, ATM, phospho-p53, p53, GADD45α, and p21 in UVB-exposed cells post-incubated with various concentrations of RA by western blot analysis (Figure 3). A threefold increase in p-ATM and p-p53, and 2.5 fold rise in GADD45α and p21 protein expression levels were observed in cells exposed to UVB and post-incubated in growth medium, as compared to that in non-irradiated control cells. The increased p-ATM, p-p53, GADD45α and p21 protein expression levels

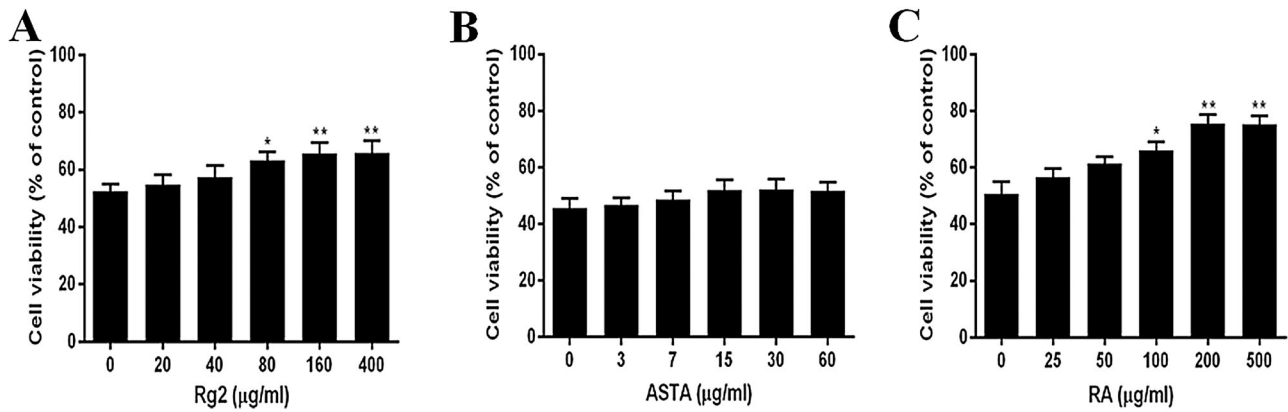


Figure 1. Effects of various concentrations of Rg2, ASTA and RA on viability of UVB-exposed HaCaT cells. Cells exposed to 700 J/m² UVB were post-incubated in growth medium or medium containing various concentrations of (A) Rg2, (B) astaxanthin, or (C) their 5:1 combination (RA) for 24 h. The cell viability was determined by MTT assay. Data shown represent the mean values of three independent experiments \pm SD. * p < 0.05 and ** p < 0.01 versus untreated UVB-exposed group.

decreased significantly in cells treated with RA post UVB exposure (Figure 3). The present results corroborate those of previous studies.

COL1A1 is the main structural protein in the extracellular space in different tissues (Di Lullo et al. 2002). They are degraded by matrix metalloproteinase (MMP) enzymes, which are increased as a result of exposure to the injury source. UV light induces the expression of MMP-1, -2, and -9 in human epidermis *in vivo* (Chung 2003), and MMP inhibition may be a strategy to prevent photo-aging (Moon et al. 2008). MMP protein acts as a primary mediator between UVB-induced skin damage and skin aging or wrinkle formation (Brennan

et al. 2003; Dong et al. 2008). Chronic UVB exposure has been reported to increase skin MMP-2 levels, as measured by gelatin zymography (Inomata et al. 2003).

To confirm the effect of RA on the expression levels of skin aging-related marker proteins, we determined the expression levels of MMP-3, -9, -13 and COL1A1 by western blot analysis (Figure 4). An approximate 2~3 fold increase in the expression level of MMP-3, -9 and -13 was observed in cells exposed to UVB and post-incubated in growth medium, as compared to the that in the non-irradiated control cells. However, COL1A1 level decreased by approximately 40% in UVB-exposed cells compared to that in the control cells. In cells exposed

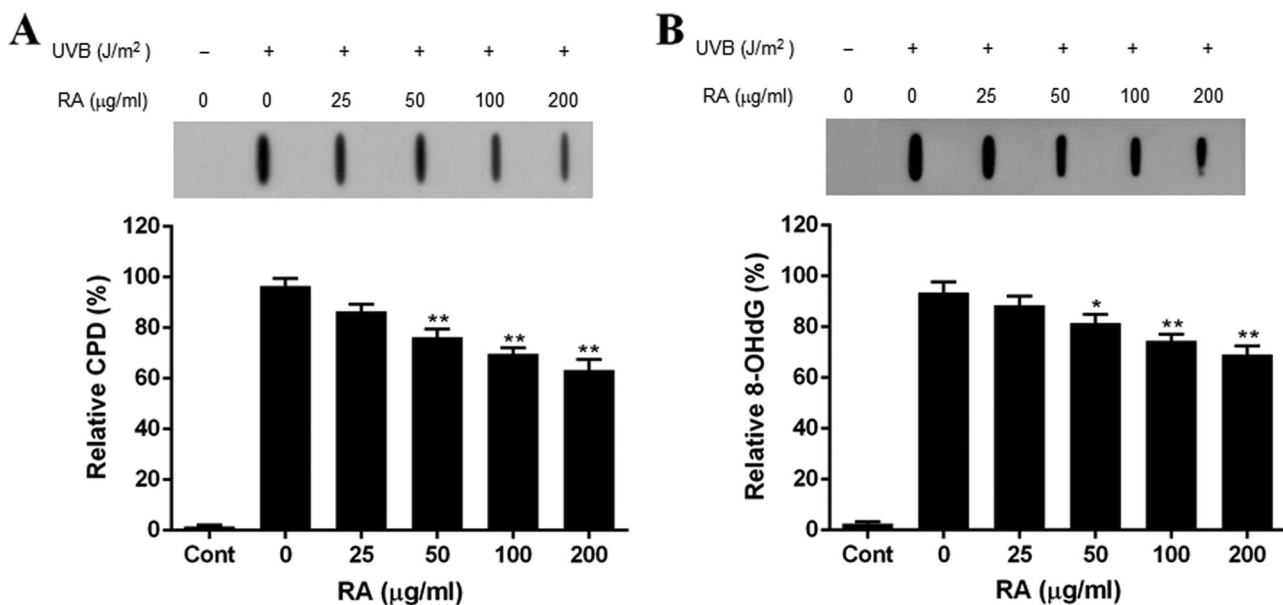


Figure 2. Effects of various concentrations of RA on the levels of CPD and 8-OHdG in UVB-exposed HaCaT cells. Cells exposed to 700 J/m² UVB were post-incubated in growth medium or medium containing various concentrations of RA for 24 h. The levels of CPD and 8-OHdG were determined by immunodot blot assay. Data shown represent the mean values of three independent experiments \pm SD. * p < 0.05 and ** p < 0.01 versus untreated UVB-exposed group (0 RA).

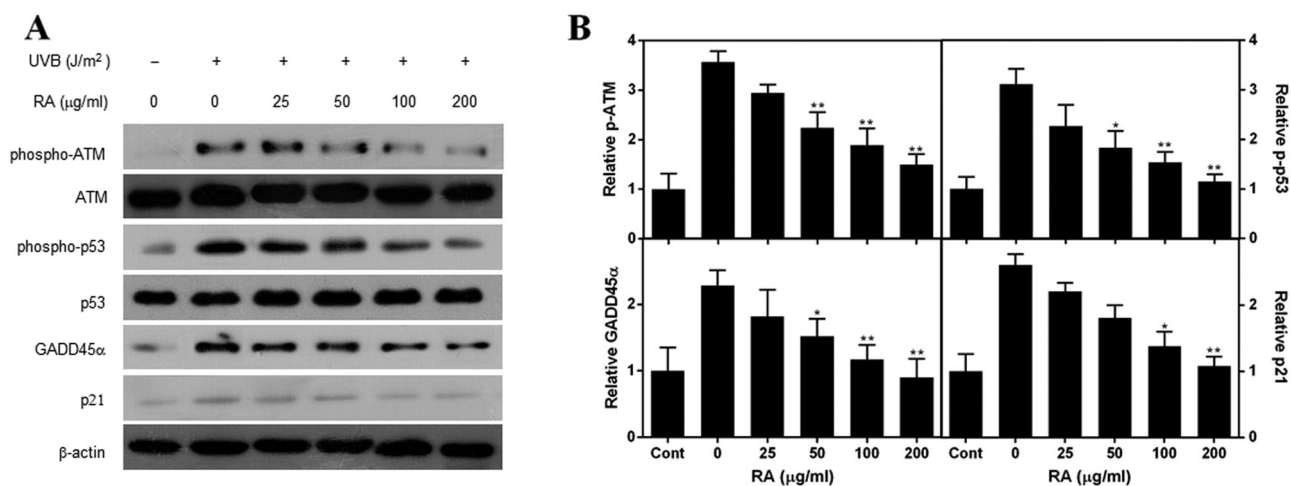


Figure 3. Effects of various concentrations of RA on the levels of DNA damage marker proteins in UVB-exposed HaCaT cells. Cells exposed to 700 J/m² UVB were post-incubated in growth medium or medium containing various concentrations of RA for 24 h. The levels of p-ATM, ATM, p-p53, p53, GADD45α and p21 were determined by western blot analysis. Data shown represent the mean values of three independent experiments ± SD. **p* < 0.05 and ***p* < 0.01 versus untreated UVB-exposed group (0 RA).

to UVB, RA treatment significantly reduced the increased MMP-3, -9, and -13 protein levels in a concentration-dependent manner. Furthermore, treating cells with RA after UVB exposure effectively recovered the decreased COL1A1 level in a concentration-dependent manner (Figure 4).

ASTA has a non-polar polyene chain at the middle of the molecule. Many studies have reported the antioxidant mechanisms of ASTA. Owing to its unique structure with polar terminal rings, ASTA can pass across cell membranes. ASTA has the ability to remove high-energy electrons from free radicals or oxidants, owing to its long carbon chain (Kidd 2011). A combination of ASTA with

α-tocopherol has been shown to reduce the levels of 8-OHdG and lipid peroxides in streptozotocin-induced diabetic rats, as compared to those in control groups (Nakano et al. 2008). ASTA has also been reported to reduce UVA-induced DNA damage in Caco-2 cells (Lyons and O'Brien 2002). Moreover, it is known to increase malondialdehyde levels and decrease DNA strand breaks. Besides, ASTA has been shown to reduce the number of TUNEL-positive cells in testicular sections of mice treated with cyclophosphamide (Tripathi and Jena 2008).

Similar to glucocorticoids, Rg2, a glucocorticoid analogue, can bind to glucocorticoid receptor (GR) and activate the GR signaling pathway. Rg2 interacts with GR to

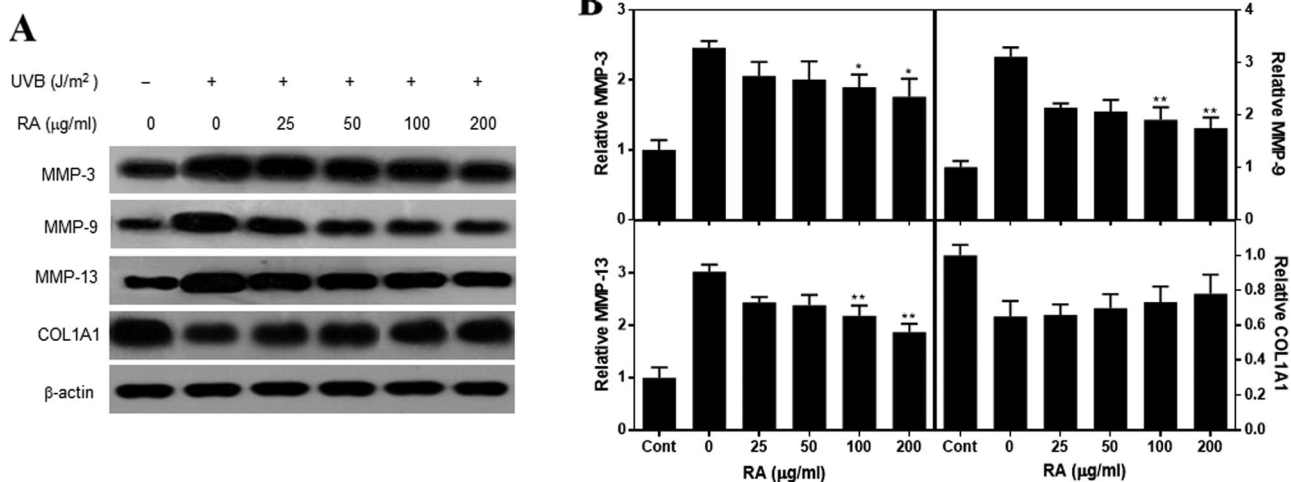


Figure 4. Effects of various concentrations of RA on the levels of photoaging markers in UVB-exposed HaCaT cells. Cells exposed to 700 J/m² UVB were post-incubated in growth medium or medium containing various concentrations of RA for 24 h. The levels of MMP-3, -9, -13 and COL1A1 were determined by western blot analysis. Data shown represent the mean values of three independent experiments ± SD. **p* < 0.05 and ***p* < 0.01 versus untreated UVB-exposed group (0 RA).

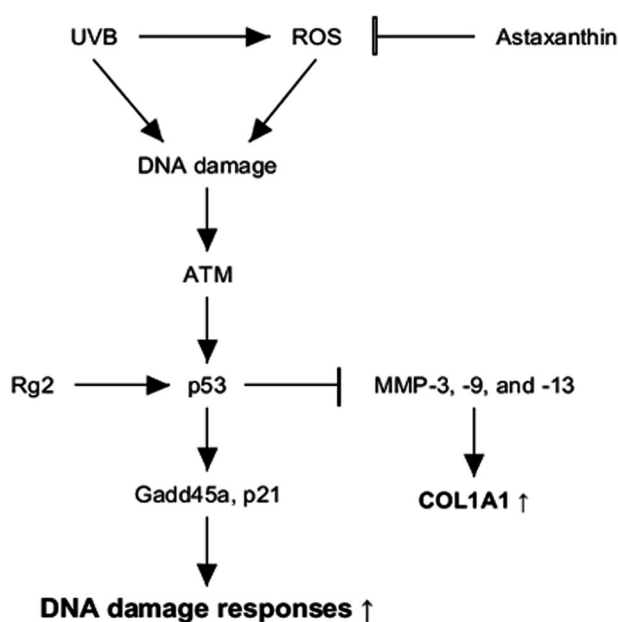


Figure 5. Proposed scheme for the effects of ASTA and Rg2 on UVB-induced DNA damage responses. ASTA, an antioxidant, decreases the level of ROS induced by UVB. Rg2, a ginseng saponin, increases the level of p53 perhaps via association with glucocorticoid receptor or directly binding to p53 at early time points after treatment and enhances the DNA damage responses through the transactivation of downstream genes, such as Gadd45a and p21.

form a homodimer and migrates into the nucleus where the GR dimer binds to the glucocorticoid receptor response element (GRE) in the promoter and induces transcriptional activation of several proteins, such as p53, thereby increasing cytoplasmic protein levels (Buckbinder et al. 1994; Hayachi et al. 2004). We previously determined that protective effects of Rg2 against UVB-induced DNA damage in HaCaT cells is dependent on p53 expression (Ha et al. 2016). Rg2-induced p53 and other proteins led cells to rapidly recover from the damage caused by extracellular environmental factors.

The UVB-induced DNA damage responses, and the possible effects of ASTA and Rg2 are schematically depicted in Figure 5. UVB induces DNA damage responses (DDR) through the activation of ATM and subsequent p53 phosphorylation. Phosphorylated p53 translocates into the nucleus and regulates transcription of genes encoding Gadd45a, p21, MMP-3, -9, and -13 (El-Deiry et al. 1993; Carrier et al. 1994; Sun et al. 1999, 2000; Ala-aho et al. 2002). p53 increases the protein levels of Gadd45a and p21 and decreases those of MMP-3, -9, and -13 (Figure 5). We suggest that ASTA decreases UVB-induced reactive oxygen species (ROS) level, and Rg2 accelerates DDR by increasing the level of p53 at early time points after treatment (Jeong et al. 2007).

Both the antioxidant activity of ASTA and the increased p53 level at early time points after exposure to Rg2 would enhance DDR, which results from the sequential actions of phospho-p53, p21, and GADD45a. The early upregulation of DDR by RA would decrease the levels of UVB-induced DNA damage and increase cell viability and COL1A1 levels at later time points (24 h) (Figures 1–3).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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