Effect of *Astilbe koreana* on Ultraviolet B (UVB)-Induced Inflammatory Response in Human Keratinocytes

MinKyun Na,\(^a,^c\) Byung Sun Min,\(^b\) Ren Bo An,\(^b\) Kyung Sik Song,\(^c\) Yeon Hee Seong,\(^d\) and KiHwan Bae*\(^e,^a\)

\(^a\) College of Pharmacy, Chungnam National University; Daejeon—705, Korea; \(^b\) Laboratory of Immunomodulator, Korea Research Institute of Bioscience and Biotechnology; Daejeon—305, Korea; \(^c\) Division of Applied Biology and Chemistry, College of Agriculture and Life Science, Kyungpook National University; Daegu—702, Korea; \(^d\) College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University; Cheongju—361, Korea; and \(^e\) Jakwang Research Institute, Hansaeng Cosmetics Co., Ltd.; Nonsan—320, Korea.

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The EtOH extract from the rhizomes of *Astilbe koreana* (Saxifragaceae) exhibited potent antioxidant activity in our recent study. Since the oxidative stress is known to be involved in the inflammatory response after ultraviolet B (UVB) exposure, the ability of the *A. koreana* extract to inhibit UVB-induced prostaglandin E\(_2\) (PGE\(_2\)) and nitric oxide (NO) production was examined. UVB irradiation (35 mJ/cm\(^2\)) increased PGE\(_2\) and NO production, which were significantly decreased by pre-administration of the *A. koreana* extract in a dose-dependent manner. The *A. koreana* extract also preserved cellular antioxidant capacity after UVB irradiation, which was determined by glutathione (GSH) content. UVB irradiation enhanced the formation of ROS in the keratinocytes, which was determined using a 2,7'-dichlorofluorescin diacetate (DCFH-DA), a redox sensitive dye. The levels of intracellular reactive oxygen species (ROS) were also significantly reduced by pretreatment of the *A. koreana* extract in a dose-dependent manner measured 9 h after UVB irradiation. The results suggest that the *A. koreana* extract may have a protective effect on the UVB-injured keratinocytes by inhibiting PGE\(_2\) and NO production, possibly through the inhibition of intracellular ROS accumulation.

Key words *Astilbe koreana*; Saxifragaceae; ultraviolet B (UVB)-induced prostaglandin E\(_2\) (PGE\(_2\)) production; UVB-induced nitric oxide (NO) production; intracellular reactive oxygen species (ROS)

It is well known that ultraviolet B (UVB, 290—320 nm) radiation is responsible for the cutaneous damage by both acute and chronic exposure, and is believed to be an important etiology in human skin cancer and premature skin aging.\(^1\)\(^,^2\) Exposure to UVB irradiation induces the inflammatory response characterized by increased blood flow and vascular permeability that result in edema and erythema, the infiltration of neutrophils into the dermis, and the introduction of pro-inflammatory cytokines.\(^3\) The primary mediator of erythema in the human skin is known to be prostaglandin E\(_2\) (PGE\(_2\)), and a specific inhibitor of prostaglandin synthesis has been suggested to reduce the UVB-induced erythema.\(^4\)—\(^6\) Together with PGE\(_2\), nitric oxide (NO) is believed to be an important inflammatory mediator of UVB irradiation acting within the epidermal skin. NO contributes to erythema by induction of vascular dilatation, and it reacts with superoxide radical to form peroxynitrite, which can influence the synthesis and activity of phospholipase.\(^7\)\(^,^8\) In association with the inflammatory response, recent studies have suggested that reactive oxygen species (ROS) are involved in UVB-induced inflammation.\(^3\)\(^,^9\) UVB irradiation induces the formation of ROS and depletes cellular antioxidant stores.\(^10\)—\(^12\) The UVB-induced oxidative stress has been implicated in the activation of various signaling pathways involved in the inflammation and cancer.\(^13\)—\(^15\) Recent studies have demonstrated that antioxidants may reduce the UVB-induced skin damage.\(^12\)—\(^14\)\(^,^15\)

*Astilbe koreana* Nakai belongs to Saxifragaceae and is a perennial herb growing in the moist fields and mountains, usually to a height of 60 cm. The rhizomes of the genus *Astilbe* including *A. koreana* have been used to treat pain, headache, arthralgia, chronic bronchitis and inflammation.\(^16\)

In our recent study, the EtOH extract from the rhizomes of *A. koreana* exhibited potent antioxidant activity on free radicals and lipid peroxidation.\(^17\) Since many harmful effects of UV radiation, including erythema, carcinogenesis and premature skin aging, are associated with the generation of ROS,\(^9\)\(^,^13\)\(^,^18\) it was of interest to examine the potential application of the *A. koreana* extract as a protective agent against UVB-induced skin damage. In this study, we examined the effect of this extract on UVB-induced PGE\(_2\) synthesis, NO production and intracellular oxidative stress in human keratinocytes.

**MATERIALS AND METHODS**

**Plant Material** The rhizomes of *A. koreana* were collected at Mt. Sulak, Korea in July 2001. A voucher specimen (CNU 0880) was identified by Prof. KiHwan Bae and deposited in the herbarium of the College of Pharmacy, Chungnam National University. Twenty grams of the dried rhizomes of *A. koreana* were extracted using 100 ml EtOH at room temperature for one month. The EtOH extract was concentrated and dissolved in DMSO or a medium for bioassay.

**Cell Culture** The human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) were purchased from Modern Tissue Technologies, Inc. (MC1312, Korea). The keratinocytes were cultured in a type IV collagen coated plate with KGM\(^8\) Bulletkit medium (Clonetics, San Diego, CA, U.S.A.) in a humidified atmosphere of 5% CO\(_2\)/95% air at 37 °C, and cultured to 90% confluence.

**UVB Irradiation** The keratinocytes grown to the logarithmic phase were seeded at a density of 1 × 10\(^4\) cells/well in a 96 well microplate to allow the cells to proliferate to the subconfluent state upon UVB irradiation. After 18 h, the *A.*
**koreana** extract was administered for 2 h. After rinsing with PBS, the cells were irradiated with a TFX-20 M UV transiluminator (VILBER- LOURMAT, France; wavelength 290—330 nm with a peak at 312 nm). UVB was irradiated with a dose of 35 mJ/cm² and monitored with an UVImeter RX 003 radiometer (UVtec, Ltd., U.K.). The UVB-irradiated cells were cultured in fresh medium for the indicated time for further experiments.

**Determination of PGE₂** The media was collected and the amount of PGE₂ was determined using an enzyme immunoassay kit, Prostaglandin E₂ EIA kit (Cayman Chemical, U.S.A.).

**Determination of Nitric Oxide (NO)** NO can be spectrophotometrically assayed by measuring the accumulation of its stable degradation products, nitrate and nitrite. For accurate assessment of the total NO generated, nitrate was converted into nitrite by enzyme nitrate reductase, followed by quantitation of nitrite using Griess reagent. NO was estimated by means of the total nitrite formed using a colorimetric NO assay kit (Oxford Biomedical Research, U.S.A.).

**Determination of Intracellular Glutathione (GSH)** The content of intracellular GSH (reduced form) was determined using a colorimetric assay kit, Bioxytech® GSH-400 (OXIS International Inc., U.S.A.).

**Determination of Intracellular ROS (DCF Assay)** The UV-irradiated cell cultures were treated with 10 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, U.S.A.) in PBS for 30 min. Thereafter the medium was discarded and the cells washed with PBS. The fluorescence intensity was determined using a CytoFluor 2350 fluorescence plate reader (Millipore) at 485 nm for excitation and 535 nm for emission. UVB-irradiated intracellular ROS was also confirmed by fluorescence microscopy examination (original magnification 1:200).

**Statistical Analysis** The data is expressed as mean±S.D., and the differences between the control and test groups were analyzed using a two-tailed Student’s t-test. Differences of p<0.05 were considered to be statistically significant.

**RESULTS**

**Effect on UVB-Induced PGE₂ Production** The keratinocytes exposed to a UVB irradiation dose of 35 mJ/cm² showed a significant induction of PGE₂ production in a time dependent manner up to 24 h. The level of PGE₂ in the culture media was significantly increased to 2225.0±141.2 pg/ml when it was measured 24 h after irradiation, whereas the level was decreased to 1043.7±118.0 pg/ml (p<0.001) when media were treated with the **A. koreana** extract at a concentration of 50 μg/ml. There was also a dose-dependent relationship (Fig. 1), suggesting that the **A. koreana** extract protected the cells from UVB-induced inflammation. UVB-induced PGE₂ production was also significantly inhibited by N-acetylcysteine (NAC) (1218.8±112.7 pg/ml, p<0.001), a positive control, at 50 μg/ml. Unirradiated control did not affect PGE₂ production (403.1±24.0 pg/ml).

**Effect on UVB-Induced NO Production** UVB exposure to the keratinocytes induced the production of NO: the generation of NO with exposure to UVB at 12 h was 235.0±11.6 μM, as compared with 16.9±3.0 μM for unirradiated control. Treatment with the **A. koreana** extract before UVB exposure was found to significantly inhibit UVB-induced production of NO at 12 h as 220.1±10.9, 188.9±8.5, 150.9±5.2 and 140.9±7.4 μM (p<0.001), respectively, at a concentration of 6.25, 12.5, 25.0 and 50.0 μg/ml (Fig. 2).

**Effect on UVB-Induced Oxidative Stress** In order to explore the relevant cellular events that may be involved in the UVB-induced inflammation, the level of intracellular oxidative stress was measured. As shown in Fig. 3, the level of intracellular GSH in UVB irradiated keratinocytes at 9 h was also significantly reduced to 28.0±2.7% compared to that of unirradiated control (p<0.001). Treatment with the **A. koreana** extract (6.25, 12.5, 25.0, 50.0 μg/ml) significantly inhibited the intracellular GSH depletion to 30.6±1.2, 31.4±1.7, 36.5±4.0 and 59.3±3.1%, respectively. NAC inhibited the intracellular GSH depletion to 35.5±2.8 and 57.2±3.2%, respectively, at 10.0 and 50.0 μg/ml. As shown in Figs. 4A and B, the level of intracellular ROS which appeared bright (yellow color) in fluorescence microphotography was significantly increased when measured 9 h after irradiation.
A. koreana extract-treated cells were 12.5, 21.7, 47.3 and 67.2% lower, respectively, than that of the control at a concentration of 6.25, 12.5, 25.0 and 50.0 μg/ml. The fluorescence intensity of the A. koreana extract at 50.0 μg/ml. The fluorescence intensity of the A. koreana extract-treatment was 12.5, 21.7, 47.3 and 67.2% lower, respectively, than that of the control at a concentration of 6.25, 12.5, 25.0 and 50.0 μg/ml. The fluorescence intensity of the A. koreana extract at 50.0 μg/ml. The fluorescence intensity of the A. koreana extract at 50.0 μg/ml.

DISCUSSION

The acute UVB-induced inflammatory response in the skin is characterized by the induction of COX-2 gene expression, with the subsequent production of prostaglandins, including PGE2, resulting in erythema and the infiltration of activated neutrophils into the dermis. Recent studies have suggested that the primary mediator of erythema in human skin is PGE2, and a specific inhibitor of prostaglandin synthesis is effective in inhibiting UVB-induced inflammation. In this study, exposure of the keratinocytes to UVB irradiation (35 mJ/cm2) significantly increased the level of PGE2 24 h after irradiation, whereas the levels of PGE2 were decreased in a dose-dependent manner when they were treated with the A. koreana extract before UVB irradiation. The result suggests that this extract can protect the keratinocytes from UVB-induced inflammation. Since edema or swelling which results from an increase in vascular permeability is one of the first physical signs of an acute UVB-induced cutaneous inflammatory response, NO is believed to involve UVB-induced inflammation via its vasodilatory action. NO also reacts with superoxide radical to form peroxynitrite, which can influence the synthesis and activity of phospholipase. Treatment with the A. koreana extract before UVB exposure was found to inhibit UVB-induced NO production in the keratinocytes. The result suggests there is another possible mechanism to the anti-inflammatory action of the A. koreana extract.

In association with the inflammatory response, recent studies have suggested that ROS is involved in UVB-induced inflammation. Exposure to UVB irradiation is known to induce excessive generation of ROS in the skin, as well as to drastically decrease the level of cellular antioxidants including glutathione. ROS mediates synthesis and phosphorylation of phospholipase A2, leading to PGE2 production after UVB irradiation. In addition, ROS is implicated in the activation of nuclear factor-κB (NF-κB), which induces the transcription of inflammatory cytokines and cyclooxygenase 2. UVB-induced ROS is also regarded to be an important mediator of a variety of cutaneous pathologies, such as cancer and skin aging. Considering this, antioxidants have been suggested to be a preventive potential agent against the inflammatory and carcinogenic effects of UVB irradiation. Since the A. koreana extract was found to have potent antioxidant activity on free radicals and lipid peroxidation in our recent study, it was of interest to examine the protective effect on the UVB-induced oxidative stress. GSH is known to be an endogenous defense mechanism against UV-induced ROS. It directly scavenges radicals by hydrogen transfer, and acts as cofactor for the enzyme GSH-peroxidase which scavenges peroxide. Moreover, GSH can react with NO to form S-nitrosoglutathione, a vasodilator compound, suggesting that the reduction in endogenous GSH affects UVB-induced inflammation. Based on the role of GSH in the UVB-induced damage, we monitored the GSH levels as a first index of intracellular oxidative stress. The level of intracellular GSH in UVB-irradiated keratinocytes was significantly reduced to 28.0±2.7% compared to unirradiated control (p<0.001). Treatment with the A. koreana extract before UVB exposure was found to inhibit UVB-induced GSH depletion, suggesting that the A. koreana extract protected the

Fig. 3. Inhibition of UVB-Induced Intracellular GSH Depletion by A. koreana Extract in Human Epidermal Keratinocytes

Values are expressed as means±S.D. of triplicate experiments. UVB irradiation significantly decreased the level of GSH compared to unirradiated control (p<0.001), and significant comparative difference is indicated by *p<0.05 or **p<0.001.

Fig. 4. Inhibition of UVB-Induced ROS Production by A. koreana Extract in Human Epidermal Keratinocytes

Fluorescence microscopy analysis of intracellular DCF formation (A) and relative DCF fluorescence intensity (B). Values are expressed as means±S.D. of triplicate experiments. UVB irradiation significantly increased the level of ROS compared to unirradiated control (p<0.001), and significant comparative difference is indicated by *p<0.05 or **p<0.001.

whereas this level was decreased when treated with the A. koreana extract at 50.0 μg/ml. The fluorescence intensity of the A. koreana extract-treated cells was 12.5, 21.7, 47.3 and 67.2% lower, respectively, than that of the control at a concentration of 6.25, 12.5, 25.0 and 50.0 μg/ml (Fig. 4B). NAC (50.0 μg/ml) also reduced DCF fluorescence formation to 60.3% that of the control.
keratinocytes from UVB-induced oxidative stress. Since GSH depletion can induce ROS accumulation, we examined in parallel the intracellular oxidative stress using DCFH-DA, a redox sensitive dye. The non-polar, non-fluorescent DCFH-DA is able to diffuse through the cell membrane and to be deacetylated by cytosolic esterase to form the polar, non-fluorescent dichlorodihydrofluorescein (DCFH). By reacting with ROS, it gives rise to the formation of the fluorescent derivative dichlorofluorescein (DCF). The accumulation of DCF, which represents the level of intracellular ROS, was significantly increased when measured 9 h after irradiation (Fig. 4), whereas the accumulation was decreased in a dose-dependent manner when treated with the *A. koreana* extract. The results thus demonstrate that the extract is capable of protecting the keratinocytes against UVB-induced oxidative damage.

In conclusion, we demonstrated that the *A. koreana* extract has an inhibitory effect on UVB-induced inflammation in human keratinocytes by blocking PGE2 and NO production; this effect can be explained, at least in part, by its inhibition of intracellular oxidative stress following UVB irradiation. In view of the results, the *A. koreana* extract might be a potential source for modulation of the deleterious effects caused by UVB exposure. The constituent responsible for the biological activities will be identified in further studies.

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**REFERENCES**