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Inhibition of UVA irradiation-modulated signaling pathways by rutaecarpine, a quinazolinocarboline alkaloid, in human keratinocytes

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Abstract

Matrix metalloproteinases (MMPs), a key component in photoaging of the skin due to exposure to ultraviolet A, appear to be increased by ultraviolet A irradiation-associated generation of reactive oxygen species. In this study, we investigated the effects of synthetic rutaecarpine, which is also found in *Evodia rutaecarpa*, on the ultraviolet A-induced changes in the expression of gelatinases: matrix metalloproteinase (MMP)-2 and MMP-9 using HaCaT human keratinocytes as a model cellular system. Ultraviolet A irradiation of HaCaT cells increased the gelatinolytic activities of MMP-2 and MMP-9, which was significantly suppressed by the pretreatment with rutaecarpine. In addition, rutaecarpine significantly suppressed the ultraviolet A-induced enhanced expression of MMP-2 and MMP-9 proteins and mRNAs. Rutaecarpine also inhibited the H_2O_2-induced increase in the expression of MMP-2 and MMP-9. Furthermore, rutaecarpine decreased the ultraviolet A-induced increased generation of reactive oxygen species. Taken together, these results suggest that rutaecarpine inhibited ultraviolet A-induced reactive oxygen species generation, resulting in the enhanced expression of MMP-2 and MMP-9 in human skin cells. These results further suggest that ruetaecarpine may be useful in the prevention of ultraviolet A-induced photoaging.

Keywords: Rutaecarpine; Reactive oxygen species; Matrix metalloproteinase; HaCaT human keratinocyte

1. Introduction

Long-term exposure to sunlight causes various detrimental effects on the skin including skin cancer and premature aging (photoaging), which is mostly caused by ultraviolet fraction. Ultraviolet A and B are responsible for the photoaging of skin characterized as wrinkles, leathery texture and mottled pigmentation (Gilchrest and Yaar, 1992; Scharffetter-Kochanek et al., 1997). Molecular mechanisms of skin wrinkles are probably due to the loss of macromolecules making up the dermal matrix (Gilchrest, 1996; West, 1994), among which collagen is the major component. The level of collagen in normal skin is maintained by the balance between synthesis by dermal fibroblasts and enzymatic degradation. In ultraviolet-irradiated skin, the level of matrix metalloproteinases (MMPs), important enzymes for the proteolysis of extracellular matrix proteins, is elevated long before the visible symptoms of photoaging (Fisher et al., 1996, 1997). Among them, gelatinases, MMP-2 and MMP-9 are synthesized as proenzymes and play an important role in degrading type IV collagen (Birkedal-Hansen et al., 1999; Johnson et al., 1998). Inhibition of induction of MMPs has been reported to alleviate ultraviolet-induced photoaging by preventing from collagen destruction (Kobayashi et al., 1998; Oikarinen et al., 1993).

It has been known that reactive oxygen species are generated in ultraviolet A-irradiated human skin, resulting in oxidative damage to lipids, proteins and DNA (Cunningham et al., 1985; Hanson and Clegg, 2002; Vile and Tyrrell, 1995). Reactive oxygen species produced by ultraviolet irradiation regulate gene expression, including induction of MMPs through redox-dependent activation of NF-κB

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(Shang et al., 2002; Scharffetter-Kochanek et al., 2000; Wenk et al., 2001).

Rutaecarpine is one of quinazolinocarboline alkaloids isolated from *Evodia rutaecarpa* that has long been utilized as a Chinese herbal medicine. It has been reported that rutaecarpine has various biological effects such as vasodilation (Chiou et al., 1994), antithrombosis (Sheu et al., 2000) and antiinflammation (Woo et al., 2001; Moon et al., 1999). Evodiame, another quinazolinocarboline alkaloid from *E. rutaecarpa*, and rutecarpine have been studied in tumor invasion and metastasis using reconstituted basement membrane and animal model (Ogasawara et al., 2002). However, to our knowledge, effects of rutaecarpine on ultraviolet irradiation-induced reactive oxygen species production and expression of MMPs have not been reported yet.

Thus, in the present study, we investigated the effects of rutaecarpine on the ultraviolet A-induced production of reactive oxygen species and expression of MMPs using HaCaT human keratinocytes as a model cellular system. We also compared their effects with those of retinoic acid, a well-known anti-wrinkle agent (Fisher et al., 1999).

2. Materials and methods

2.1. Materials

Rutaecarpine was synthesized according to our previous report (Lee et al., 2001). The powders for Eagle's minimum essential medium, trypsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium pyruvate and all salt powders were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum and antibiotics were purchased from Gibco (Grand Island, NY, USA). The stock solutions of drugs were sterilized by filtration through 0.2-μm disc filters (Gelman Sciences, Ann Arbor, MI, USA).

2.2. Cell culture and treatment

HaCaT cells (Boukamp et al., 1988) were grown at 37 °C in a humidified incubator under 5% CO_2/95% air in MEM supplemented with 10% fetal bovine serum, 200 IU//ml penicillin, 200 mg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence, the cells were subcultured following trypsinization. Cells were irradiated using a ultraviolet radiator (Lilber Lourmat, France) emitting wavelength 365 nm for 4 min, of which dose were set at 320 mJ/cm^2. Following irradiation, the cells were incubated for further 12 h.

2.3. Cell viability assay (MTT staining)

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining method (van de Loosdrecht et al., 1991). Cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In the control experiments, cells were grown in the same media containing drug-free vehicle. After incubation with the drug for 48 h, 100 μl of MTT (5 g MTT/l in H_2O) were added and cells incubated for a further 4 h. A 200-μl sample of dimethyl sulfoxide was added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an enzyme-linked immunosorbent assay reader (Molecular Devices, Menlo Park, CA) with a 540-nm filter.

2.4. Zymography

Enzyme activity of MMP-2 was assayed by gelatin zymography according to the method of Herron et al. (1986). The conditioned media of 1×10^4 cells were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel containing 0.33 mg/ml gelatin. The electrophoresed gel was washed twice with washing buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 2.5% triton X-100, followed by a brief rinsing in washing buffer without triton X-100. Then, the gel was incubated with incubation buffer of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 and 1 μM $ZnCl_2$ at 37 °C. After incubation, the gel was stained with 0.25% Coomassie blue R250 and destained with 7% acetic acid. Presence of the MMP was identified with a clear zone of gelatin digestion. The density of bands for pro-MMP-2 and MMP-2 were measured by using Image analyzing system (UVP, Upland, USA).

2.5. Measurement of MMP-2 and MMP-9 secretion

Secreted portions of MMP-2 and MMP-9 were measured in aliquots from the cell culture media of keratinocytes having the same cell density and incubated under various conditions using enzyme-linked immunosorbent assay systems (Oncogene QIA63-1EA for MMP-2 and Oncogene QIA56-1EA for MMP-9) according to manufacturer's instruction.

2.6. Western blot analysis

Cells were washed with a phosphate-buffered saline and centrifuged at 1000×*g* for 5 min. Cell pellets were lysed for 15 min at 4 °C in whole-cell extraction buffer containing 50 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl floride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 40 mM α-glycerophosphate and 0.1 mM dithiothreitol. Lysates were centrifuged at 20,000×*g* for 10 min at 4 °C, and the concentration of the supernatant proteins were determined by using Bradford protein assay kit (Sigma). Equal amount of the proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel

and transferred to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England) at 30 V for overnight. The membrane was blocked with 5% skim milk in Tween-20 containing Tris-buffered saline (TTBS) (20 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.05% Tween-20) and incubated with primary anti-human MMP-2 (Oncogene Research Products, Boston, USA) with dilution of 1:2000 or MMP-9 (Oncogene Research Products) antibody with dilution of 1:3000 in TTBS containing 5% skim milk. After incubation with horseradish peroxidase-conjugated anti-immunoglobulin G antibody (Santa Cruz Biotechnology, CA, USA) with dilution of 1:2000, immunodetected proteins were visualized by using enhanced chemiluminescence assay kit (Amersham Life Science). Equal loading of the samples were assured by the staining of protein-transferred membrane with ponceau S. The density of bands for MMP-2 and MMP-9 was measured by using Image analyzing system (UVP).

2.7. Reverse transcription-PCR analysis

Total cellular RNA was isolated using TRIzol reagent (Life Technologies, MD, USA) according to the manufacturer's instructions. The production of cDNA was carried out using a ready-to-go T-primed first strand kit (Amersham Biosciences). The polymerase chain reaction was performed using the primers specific for human MMP-2 (forward: 5′-GTGCTGAAGGACACACTAAAGAAGA-3′, reverse: 5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′), MMP-9 (forward: 5′-CACTGTCCACCCCTCAGAGC-3′, reverse: 5′-GCCACTTGTCGGCGATAAGG-3′) and glyceraldehydes 3-phosphate dehydrogenase (forward: 5′-GGTGAAGGTCGGAGTCAACG-3′, reverse: 5′-CAAAGTTGTCATGGATGACC-3′) in the presence of a Taq DNA polymerase (SolGent, Daejeon, Korea). PCR conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, polymerization and elongation at 72 °C for 1 min and final elongation at 72 °C for 10 min. The PCR products were detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.8. Nitroblue tetrazolium reduction assay

The intracellular superoxide generation was measured by using the conversion of nitroblue tetrazolium to formazan. Nitroblue tetrazolium was added to the medium of cells to a final concentration of 1 mg/ml. After the drug treatment, cells were lysed and formazan was dissolved with 2 M KOH and 1.4 volume of dimethyl sulfoxide. The absorbance was read spectrophotometrically at 654 nm.

2.9. Data analysis

Data were expressed as mean±standard error of the mean (S.E.M.) and were analyzed using one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P*-values less than 0.05 are considered statistically significant.

3. Results

3.1. Effects of rutaecarpine on the viability of HaCaT cells

To select maximum concentrations of rutaecarpine without inducing cell toxicity, cell viability were determined using the MTT assay before examining the effects on the expression of MMPs in HaCaT cells. As shown in Fig. 1, maximum concentration without significant cytotoxic effects was 25 μM for rutaecarpine, which was higher than that for retinoic acid (1 μM), a well-known anti-wrinkle agent. Thus, we used 1 and 10 μM of rutaecarpine (in these concentrations, no significant toxic effect was observed) throughout the experiments.

3.2. Effects of rutaecarpine on the ultraviolet A-induced activation and expression of MMP-2 and MMP-9 in HaCaT cells

The effect of ultraviolet A (365 nm) irradiation on the gelatinolytic activity of MMP-2 and MMP-9 was examined using the zymographic analysis in HaCaT human keratinocytes. As shown in Fig. 2A, activation of MMP-2 and MMP-9 was increased by ultraviolet A (4 min) irradiation corresponding to 320 mJ/cm^2. Pretreatment with rutaecarpine dose-dependently suppressed the ultraviolet A-induced activation of MMP-2 and MMP-9. The effect of rutaecarpine at the concentration of 10 μM was comparable to that of retinoic acid (2 μM). The ultraviolet A-induced increased secretion of MMP-2 and MMP-9 measured by enzyme-linked immunosorbent assay was significantly inhibited by

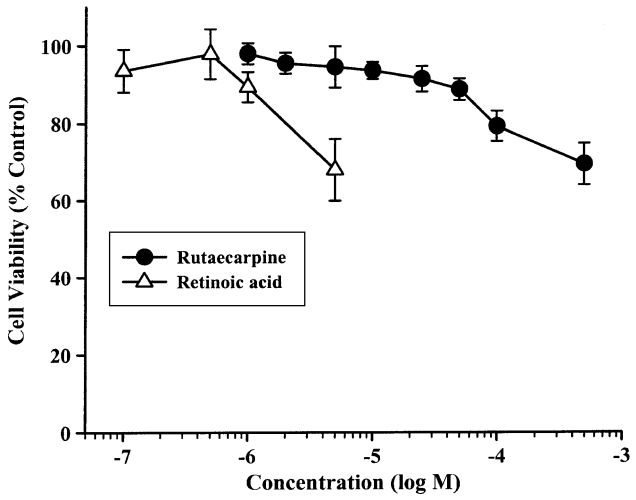

Fig. 1. The effects of rutaecarpine on the viability of HaCaT human keratinocytes. Cells treated for 24 h with or without each concentration of drugs were analyzed for viability by MTT assay.

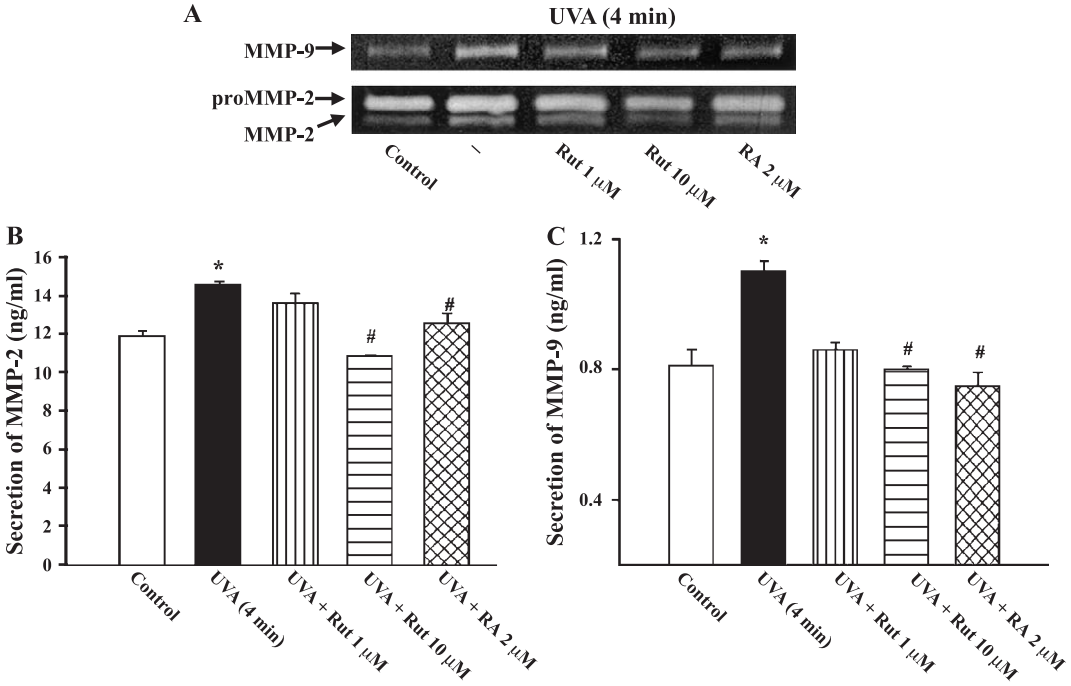

Fig. 2. Inhibitory effects of rutaecarpine on the activation of MMP-2 and MMP-9 by ultraviolet A irradiation in HaCaT cells. Cells were supplemented with PBS 24 h prior to UVA (365-nm) irradiation. The conditioned media 12 h after irradiation were analyzed for the gelatinolytic activity (A) by zymography and for the amount of MMP-2 (B) and MMP-9 (C) by ELISA method. Rut and RA represents rutaecarpine and retinoic acid, respectively.

rutaecarpine as depicted in Fig. 2B and C. Furthermore, the expression levels of MMP-2 and MMP-9 by ultraviolet A irradiation were significantly suppressed by the pretreatment with rutaecarpine (Fig. 3). The effect of rutaecarpine at the concentration of 10 μM was more effective than that of *N*-acetylcysteine (100 μM), an antioxidant, but comparable to that of retinoic acid (2 μM). In order to determine whether rutaecarpine affects MMP synthesis at the molecular level, we performed reverse transcription-PCR analysis. As shown in Fig. 4, rutaecarpine itself did not affect the mRNA level of MMP-2 and MMP-9. However, rutaecarpine prevented the ultraviolet A-enhanced expression of MMP-2 and MMP-9 mRNAs.

3.3. Inhibitiory effect of rutaecarpine on ultraviolet A-induced ROS generation and H_2O_2-induced expression of MMP-2 and MMP-9

Since reactive oxygen species are generated by ultraviolet A irradiation in the skin and it appears to play an important role in induction of MMPs, we investigated whether inhibitory effect of rutaecarpine on ultraviolet A-

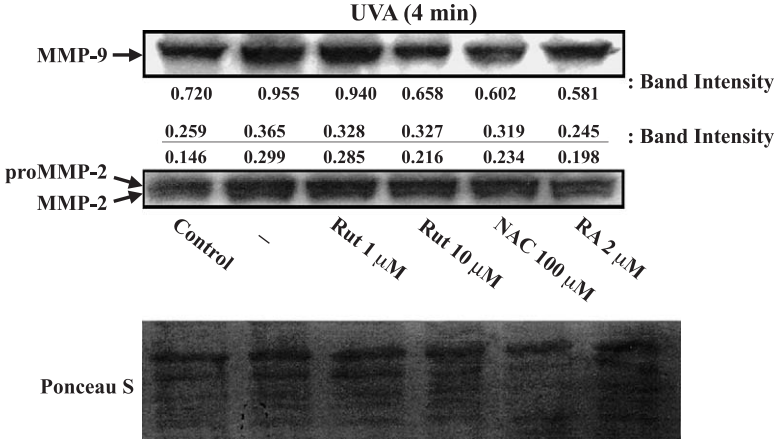

Fig. 3. Prevention of ultraviolet A-induced expression of MMP-2 and MMP-9. The cells were incubated with rutaecarpine or retinoic acid for 24 h prior to ultraviolet A irradiation for 4 min. The expression of MMP-2 and MMP-9 was measured by Western blot. Equal loading of the samples were assured by the staining of protein-transferred membrane with ponceau S. Rut, rutaecarpine; RA, retinoic acid.

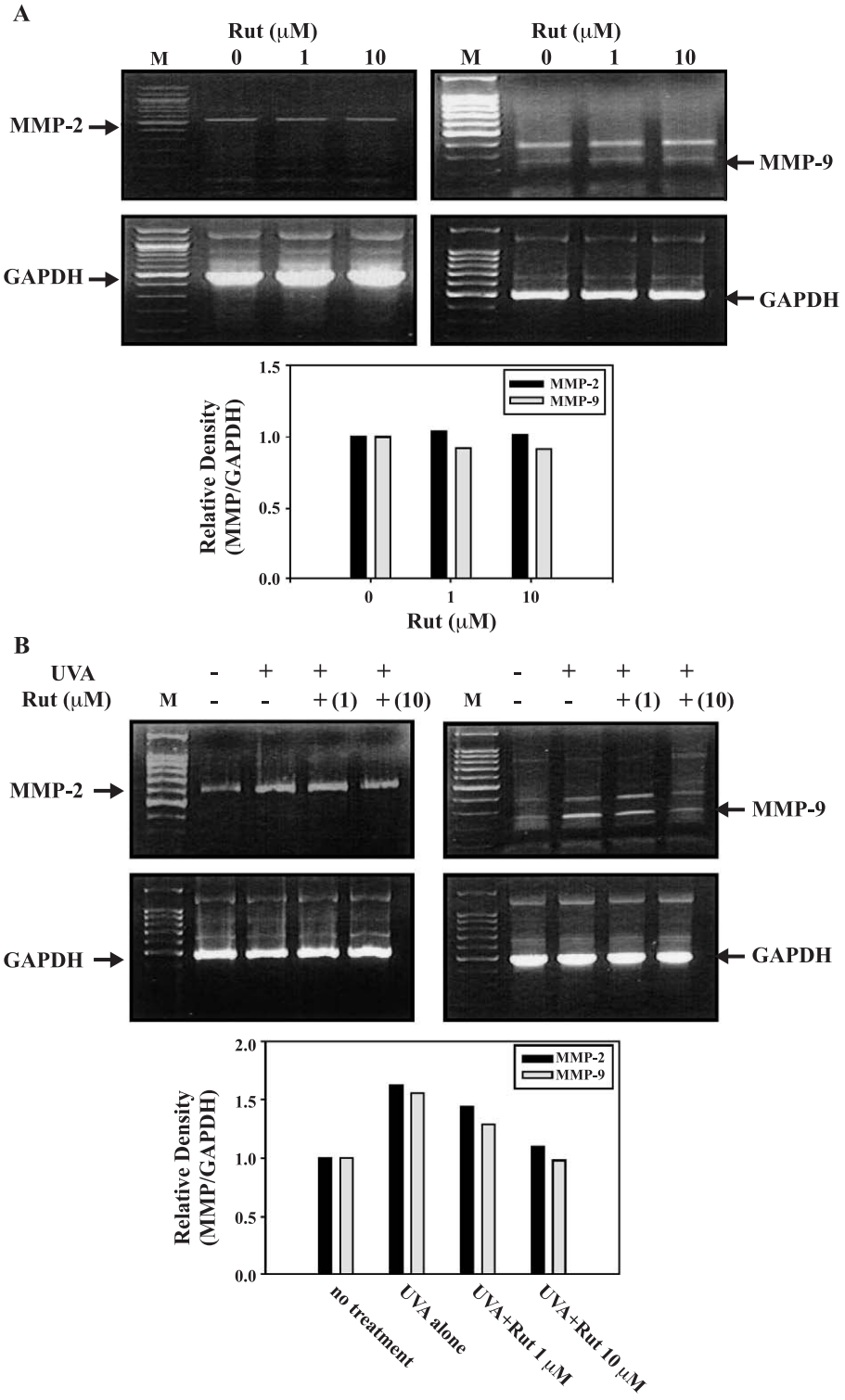

Fig. 4. Dose-dependent suppression of ultraviolet A-induced increase in the MMP-2 and MMP-9 mRNA levels by rutaecarpine in HaCaT cells. (A) Cells were only treated with rutaecarpine. (B) Cells were pretreated with different concentrations of rutaecarpine prior to ultraviolet A irradiation. DNA size markers are shown in the left lanes (M). Arrows indicate the predicted size of PCR products. In bar graphs, the data represent the relative intensity of the bands (MMP/GAPDH). GAPDH represents for glyceraldehydes 3-phosphate dehydrogenase.

modulated changes in MMPs is mediated by suppression of ultraviolet A irradiation-induced reactive oxygen species generation. Rutaecarpine significantly inhibited both the basal generation of reactive oxygen species and ultraviolet A-induced enhanced reactive oxygen species production as shown in Fig. 5. In addition, as shown in Fig. 6, exogenous application of H_2O_2 (100 μM) increased the expression of MMP-2 and MMP-9 similar to the effect of ultraviolet A

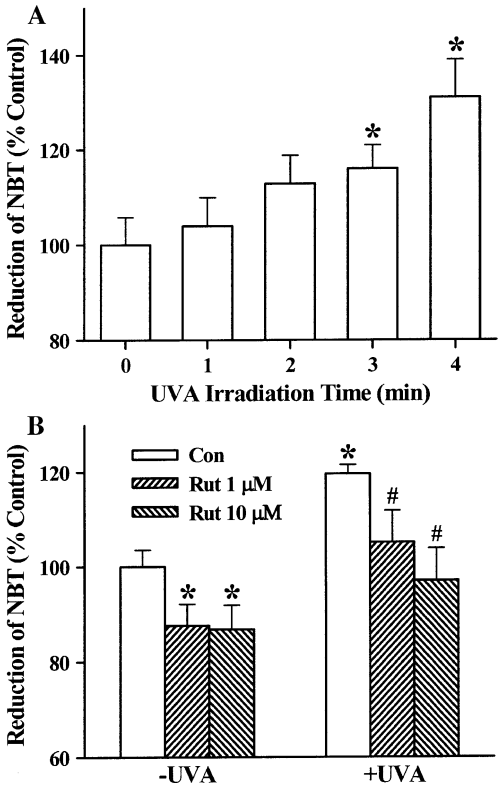

Fig. 5. Rutaecarpine suppresses the basal generation of ROS and ultraviolet A-induced production of ROS in HaCaT cells. Cells were treated for 24 h with or without drugs prior to UVA (365 nm, 3 min) irradiation. Data points represent the mean values of four replications with bars indicating S.E.M. *P<0.05 compared to control. #P<0.05 compared to UVA-irradiated group.

irradiation, which was significantly suppressed by rutaecarpine (10 μM).

4. Discussion

The studies on development of novel agents with anti-photoaging capabilities particularly from natural resources including various plants have been intensively performed. To this end, the antioxidative activities and inhibitory effects on induction of MMPs are particularly focused on since these properties appear to be major components of anti-photoaging actions. Previously, we have reported that asiatic acid and ursolic acid, triterpene compounds, possessed inhibitory activities on ultraviolet A-induced induction of MMP-2 (Lee et al., 2003). We report here that rutaecarpine, a quinazolinocarboline alkaloid, also effectively blocked ultraviolet A irradiation-modulated changes in reactive oxygen species production and induction of MMP-2 and MMP-9 in human keratinocytes.

Ultraviolet irradiation is well known to induce photodamage and premature skin aging. Since molecular mechanism of skin wrinkles seems to be due to the loss of collagen component of the dermal matrix by enhanced enzymatic degradation through the induction of MMPs (Gilchrest, 1996; West, 1994), inhibition of MMPs appears to be useful intervention for skin-wrinkle (Fisher et al., 1999). In addition, reactive oxygen species production and oxidative processes associated with UV irradiation are considered as important components of photoaging. Reactive oxygen species play a role in the ultraviolet irradiation-induced expression of MMPs through redox regulatory transcription factors (Masaki et al., 1995). In accordance with previous other reports, our data also demonstrated that ultraviolet A irradiation increased reactive oxygen species production (Fig. 5). In addition, our results showing that exogenous H_2O_2 induced MMP-2 and MMP-9 similar to that of ultraviolet A irradiation (Fig. 6) further support reactive oxygen species involvement in ultraviolet A-induced expression of MMP-2 and MMP-9. Rutaecarpine, previously reported to have anti-invasive and metastatic activities (Ogasawara et al., 2002), inhibited the ultraviolet A-induced activation (Fig. 2) and expression of MMP-2 and MMP-9 (Fig. 3). More importantly, rutaecarpine profoundly suppressed the reactive oxygen species generation induced by ultraviolet A irradiation (Fig. 5) and inhibited induction of MMP-2 and MMP-9 by exogenous H_2O_2 application (Fig. 6), implicating that these inhibitory effects of rutaecarpine on the ultraviolet A-modulated expression of MMPs may result from suppression of reactive oxygen species generation. Since reactive oxygen species play a central role in ultraviolet irradiation-mediated apoptotic cell death (Kulms

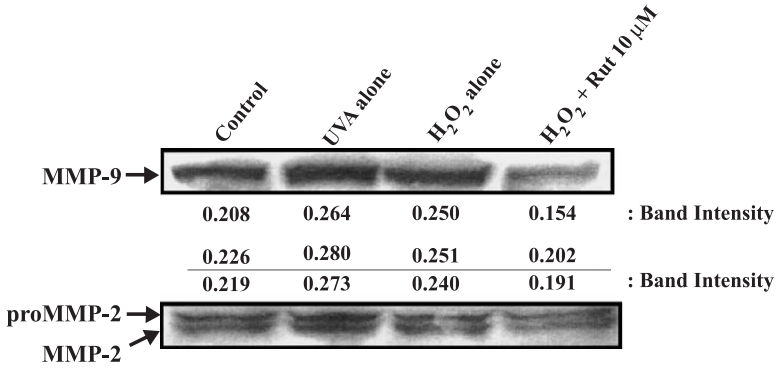

Fig. 6. Rutaecarpine prohibits enhanced expression of MMP-2 and MMP-9 by H_2O_2 application in HaCaT human keratinocytes. Cells were supplemented with PBS 24 h prior to UVA (365-nm) irradiation or H_2O_2 treatment and harvested for 12 h after the treatment. Western blot analysis was performed.

et al., 2002), these results further imply that rutaecarpine may have a protective potential against photoaging as well as reactive oxygen species-mediated photodamage of the skin.

In conclusion, rutaecarpine inhibited ultraviolet A irradiation-induced increased production of reactive oxygen species and induction of MMP-2 and MMP-9 in HaCaT human keratinocytes. These results suggest that rutaecarpine may act as an effective inhibitor of ultraviolet A-modulated signaling pathways in human skin cells. These results further suggest that rutaecarpine may be useful for the prevention of ultraviolet A-induced photoaging.

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