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Inhibition of matrix metalloproteinase-1 and -2 expression using nitric oxide synthase inhibitors in UV-irradiated human dermal fibroblasts

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Synopsis

The production of matrix metalloproteinases (MMP) by UV-irradiated skin fibroblasts and the degradation of the extracellular matrix by these enzymes is known as one of the main causes of photoaging. Recently, the Fisher group showed that MMP expression is mainly regulated by members of the mitogen-activated protein kinase family such as extracellular signal-regulated kinase, c-Jun amino-terminal kinase, and p38, each of which forms a signaling pathway. In this work, we initially examined the effect of nitric oxide (NO) and nitric oxide synthase (NOS) inhibitors on the production of MMP-1 and MMP-2 by human dermal fibroblasts (HDF). NO is a multifunctional messenger molecule generated from L-arginine and can activate guanylate cyclase to increase cGMP. We found that treatment of HDF with an NO donor, sodium nitroprusside (50 microM), enhanced the expression of MMP-1 and -2 by 153% and 243%, respectively, and treatment by 8-Br-cGMP enhanced MMP-1 and -2 expression by 137% and 254%, respectively. When UV-irradiated HDF was treated with NOS inhibitors such as aminoguanidine (AG) and baicalein (BAC), there resulted a decrease in MMP production. When 20 microM of BAC was added in the culture media of UV-irradiated HDF, only 40% of MMP-1 and 42% of MMP-2 was produced, compared to the case without BAC. Taken together, we concluded that the production of MMP-1 and -2 by UV-irradiated HDF is regulated through the signaling pathway involving NO and that it can be downregulated using NOS inhibitors.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of enzymes that are responsible for the degradation of extracellular matrix (ECM) components such as collagen, laminin, and proteoglycans. Among the MMPs, MMP-1 is an interstitial collagenase that degrades fibrillar collagens and proteoglycans, and MMP-2 is a gelatinase that degrades denatured collagens and elastin. The main reason for the connective tissue changes in the UV-irradiated skin has been clarified as the production of MMPs, including MMP-1 and MMP-2 (1–5), and the degradation of ECM components by these enzymes. The expres-

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sion of MMPs in UV-irradiated fibroblasts is known to be initiated by singlet oxygen (6), alpha-melanocyte stimulating hormone produced by keratinocytes (7), or by the activation of cell surface growth factor and cytokine receptor, which mimics the actions of receptor ligands (8,9). Recently, Fisher *et al.* (10) showed that MMP expression is mainly regulated by members of the mitogen-activated protein (MAP) kinase family such as extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38, each of which forms a signaling pathway. In some types of cells, the expression of MMP is mediated by nitric oxide (NO) (11,12). NO is a multifunctional messenger molecule generated from L-arginine by enzymes such as inducible nitric oxide synthase (iNOS), and it can activate guanylate cyclase, stimulating the production of intracellular cGMP and activation of cGMP-dependent protein kinase. The production and diffusion of NO in triggering the melanogenesis of melanocytes by the UV-irradiated keratinocytes is well documented (13), but relatively little is known about the effect of NO on the production of MMP by the epidermal fibroblasts, which are also under the influence of cytokines released from keratinocytes. In this work, we examined the effect of NO and iNOS inhibitors on the production of MMP-1 and MMP-2 by UV-irradiated human dermal fibroblasts (HDF). We found that treatment of HDF with an NO donor, sodium nitroprusside (SNP), enhanced the expression of MMP-1 and -2 and that treatment with iNOS inhibitors such as aminoguanidine (AG) and baicalein (BAC) resulted in a decrease in MMP production. Taken together, we concluded that the production of MMP-1 and -2 by UV-irradiated HDF is regulated through the signaling pathway involving NO, and that it can be downregulated using iNOS inhibitors such as AG, BAC, and the extract of Scutellaria root containing large amounts of iNOS inhibitors.

MATERIALS AND METHODS

REAGENTS

Sodium nitroprusside (SNP) (14,15), aminoguanidine (AG), nitro-L-arginine methyl ester (NAME), nitro-L-arginine (NAL), baicalein (BAC), and 8-Br-cGMP, anti-mouse IgG for the secondary antibody, were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-MMP-1 antibody (Ab-5), anti-MMP-2 antibody (Ab-3), and anti-mouse IgG conjugated with alkaline phosphatase were obtained from Cal-Biochem. Scutellaria root extract was obtained from Bioland.

CULTURE OF HUMAN DERMAL FIBROBLASTS

HDFs from newborn foreskin were acquired from Korea Cancer Center Hospital. HDFs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and kept in a humidified 5% CO_2 atmosphere were 37°C. HDFs from passages 6 to 10 were used in the experiments.

UV IRRADIATION AND NO DETECTION

HDFs (1.5×10^5/well) were seeded into 35ø plates and cultured overnight. Prior to UV irradiation, the cells were washed twice with phosphate-buffered saline (PBS). The cells were irradiated from a distance of 15 cm by a UV source (UVA simulator, Jhonsam,

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Seoul) emitting wavelengths in the range of 340–450 nm. The radiation intensity was measured using a UV radiometer (EKO, Japan). The culture medium, DMEM, containing no serum, was added and incubated for 12 hours. The concentration of MMP-1 and -2 in the culture media was determined as described below. For the determination of NO in the cell body, cells were pretreated with nitric oxide sensor dye (1 μg/ml) (Clontech; ApoAlert® nitric oxide/Annexin V dual sensor kit) and incubated for 30 minutes. The cells were UV-irradiated and incubated with the medium for 24 hours. Cells were washed twice with PBS and collected using trypsin-EDTA. The collection was centrifuged at 15,000 rpm for five minutes, and the supernatant was retained. This procedure was repeated twice. The pellet was resuspended with PBS. To analyze cells stained with NO sensor dye, a fluorescence-activated cell sorter (FACStarplus, Becton Dickinson) was used.

ZYMOGRAPHY

Zymography in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.15% gelatin was performed according to the method of Demeule *et al.* (16). The samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37°C for 20 minutes, and electrophoresed on 10% polyacrylamide gels at 4°C. After electrophoresis, the gels were washed in 2.5% Triton X-100 for one hour to remove SDS and incubated for 12 hours at 37°C in 50 mM Tris-HCl, pH 7.6; 0.15 NaCl; 10 mM $CaCl_2$; and 0.02% NaN_3, and then stained with 0.1% Coomassie Brilliant Blue R250.

DETERMINATION OF MMP-1 AND -2 BY ELISA

The expression of MMP-1 and -2 was assayed by enzyme-linked immnosorbent assay (ELISA). HDFs (8×10^3/well) were seeded into 96-well plates and cultured overnight. The culture media were replaced with DMEM containing SNP and/or AG. After incubation for 12 hours, the supernatants were transferred into a 96-well plate, and the coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) was added 1:1 (v/v) and incubated for 12 hours. The supernatants were removed and the coated well was washed with PBS-T three times, followed by blocking with 5% skim milk in PBS for one hour at 37°C. After washing three times with PBS containing 0.05% Tween 20 (PBST), 50 μl of 1/1000 diluted primary antibody, Ab-5 or Ab-3, in PBST was added into each well and incubated for 40 minutes. After washing the wells with PBST three times, 50 μl of 1/1000 diluted secondary Ab and anti-mouse IgG conjugated with alkaline phosphatase in PBST was added and incubated for 40 minutes. After washing five times with PBST, 100 μl of 1 mg/ml pNPP (p-nitrophenyl phosphate) in diethanolamine buffer was added. The optical density was measured at 405 nm after 30 minutes. The cytotoxicity of the supplemented chemicals was measured by a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

RT-PCR-ELISA

Total RNA was extracted from cultured cells using the Promega RNAgent® total RNA isolation system. PCR amplification was carried out using 5′ biotinylated primers

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(sense) to generate biotinylated PCR products detectable by digoxygenin-labeled probes in an immunoenzymatic assay (ELISA) method (17). First, cDNA was mixed with 10× buffer, 10 mM dNTPs, 1 U Taq DNA polymerase (Bioneer, Korea), and iNOS primers in a final volume of 50 µl. The primer sequences and relative predicted PCR product sizes are given below:

Human iNOS (sense) 5′-AGTTTCTGGCAGCAACGG-3′
Human iNOS (anti-sense) 5′-TTAAGTTCTGTGCCGGCAG-3′

A sample containing all reaction reagents except cDNA was used as PCR negative control in any amplification. The mixtures were incubated for the indicated cycles (predenaturation 5 min at 95°C; denaturation 50 sec at 95°C; annealing 20 sec at 56°C; extension 20 sec at 72°C) in a GeneAmp PCR System 2400 (Perkin-Elmer). The correct size of all PCR products was confirmed by comparing with a DNA standard on agarose gel.

After a given cycle of PCR, the amount of amplified cDNA was determined by the ELISA method. First, microplates (Maxisorp Nunc) were coated with 50 µg/ml of avidin (Sigma) in coating buffer (CB; 15 mM Na_2CO_3, pH 9.6) and incubated for two hours at 37°C. After incubation, free sites were saturated with 2% blocking solution (Roche, Germany) in CB. Biotinylated PCR products diluted in PBS containing 3% bovine serum albumin (PBSB) were distributed onto microplates (100 µl per well) and incubated for one hour at room temperature. After incubation, the microplates were washed three times with PBST. Amplified cDNA was denatured using 0.25 M NaOH at room temperature for ten minutes. Following the washing, 100 µl per well of 10 pmol/ml digoxygenin-labeled probes in hybridization buffer [6.25× SSC, 0.625% blocking reagent (Roche), 0.125% Tween 20, and 0.5 M NaH_2PO_4 (pH 6.5)] were added and incubated at 42°C for two hours. Anti-digoxygenin AP-conjugated antibody (Sigma) was added (1:3000 in PBSB) and incubated for one hour at 37°C. The reaction was developed by nitrophenyl phosphate (pNPP; 1M diethanolamine buffer, pH 9.6). The amount of amplified product was measured for optical density at 405 nm (OD 405) using a microplate reader.

STATISTICAL ANALYSIS

Results were presented as means ± standard error (SE). Experimental results were statistically analyzed by using Student's *t*-test (SigmaPlot 2000). *P* values <0.05 were regarded as indicating significant differences.

RESULTS AND DISCUSSION

EFFECT OF UV ON THE PRODUCTION OF MMPS

The immunoreactive MMP-1 and -2 in the culture medium of HDFs were measured using anti-MMP-1 and -2 monoclonal antibodies, respectively. Treatment of HDFs with UV radiation enhanced the production of MMP-1 by twofold and MMP-2 by threefold in a dose-related manner (Figure 1), confirming the previous results (1–5). We also confirmed that the gelatinase activities were proportionally increased by the UV irradiation of HDFs using gelatin zymography (data not shown). The production of MMPs

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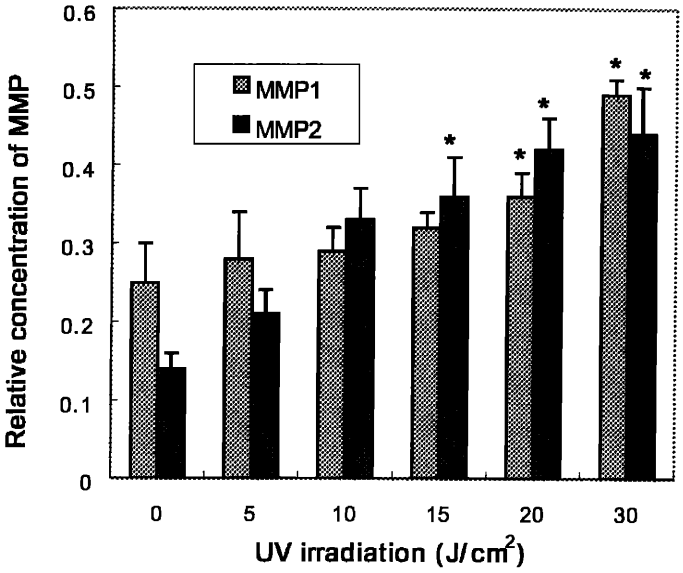

Figure 1. The effect of UV irradiation on the production of MMP-1 and MMP-2 by human dermal fibroblasts. HDFs (1.5×10^5/well) were seeded into 35ø plates and cultured overnight. The cells were irradiated from a distance of 15 cm by a UV source for a given time. *n = 3. $p < 0.05$ vs no UV exposure.

by UV irradiation of HDFs is a result of the activation of cell surface growth factors and cytokine receptors, which have in common the requirement for dimerization to initiate signal transduction. UV radiation rapidly activates EGF receptors, followed by the activation of Ras, ERK, JNK, and p38 (18,19). These stress-activated MAP kinases then increase the proteins of c-Jun, c-Fos, and ATF. The dimerization of these proteins activates AP-1 DNA binding and, finally, the induction of MMPs. Fisher *et al.* showed that retinoic acid can inhibit the induction of MMPs by UV irradiation in human skin by blocking the DNA binding of AP-1 and c-Jun protein induction (20).

EFFECT OF NITRIC OXIDE DONOR ON THE PRODUCTION OF MMPS

To determine the effect of NO on the production of MMP-1 and -2, HDFs were treated with SNP. SNP is a donor of NO and it can mimic the cellular effects of NO. Treatment with SNP increased MMP-2 production acutely, to about 243% of untreated cells with 50 microM of SNP. The amount of secreted MMP-1 in the HDF culture medium was also increased with SNP treatment, but was less significant than that of MMP-2, about 153% (Figure 2A). The result of zymography also shows that gelatinase activity was increased with SNP treatment. The combined effect of SNP and UV treatments was also tested, as shown in Figure 2B. The null hypothesis was that the effect of UV irradiation is not mediated by NO, and that subsequently the addition of SNP to UV-irradiated HDF should increase MMP production to the same degree as in cells not treated with UV irradiation and more than in cells treated only with UV irradiation. In the UV-treated cells, 50 microM of SNP did not increase MMP-1 production significantly, while that of MMP-2 increased by 196% (Figure 2B). This result indicates that the effect of UV irradiation and SNP treatment on MMP-1 production by HDF is not cumulative,

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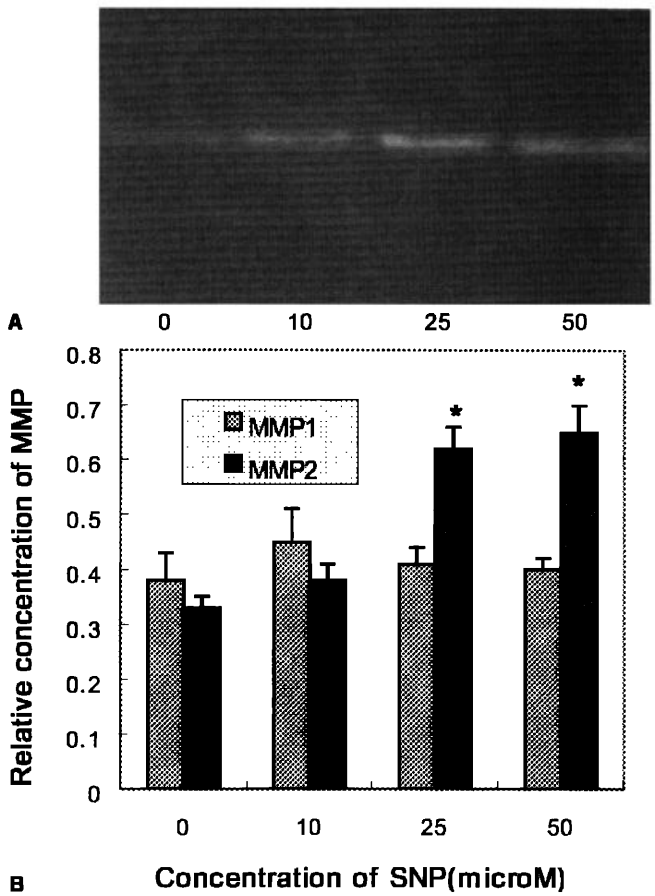

Figure 2. The effect of sodium nitroprusside on the production of MMP-1 and MMP-2 by human dermal fibroblasts. A: without UV irradiation. B: with UV irradiation. *n = 3. $p < 0.05$ vs no treatment.

which means that the UV irradiation already produced a near-maximum increase in the production of MMP-1 and the addition of 50 microM of SNP could not increase the MMP-1 production further. On the other hand, the production of MMP-2 seems to be influenced by both UV irradiation and SNP treatment at the same time. In some biological systems, the effects of NO are mediated by cGMP; this signaling cascade may involve activation of guanylate cyclase, up-regulation of cGMP, and activation of cGMP-dependent protein kinase (21). To determine the degree of involvement of cGMP in the production of MMPs, the effects of 8-Br-cGMP were measured. 8-Br-cGMP is a stable cell-permeable analog of cGMP that can mimic the cellular effects of cGMP. Treatment with 50 microM of 8-Br-cGMP also increased MMP-1 and -2 production by 137% and 254%, respectively, similar to the effect of SNP (Figure 3). This result indicates that the increase in MMP-1 and -2 production by SNP-treated HDF is not the result of a toxic effect of SNP on the cells, but is mediated by NO or related metabolites, and that NO and cGMP possibly mediate the UV-induced increase of MMP production.

EFFECT OF UV ON THE PRODUCTION OF iNOS AND NO

The production of nitric oxide in UV-irradiated keratinocytes was reported by Roméro-

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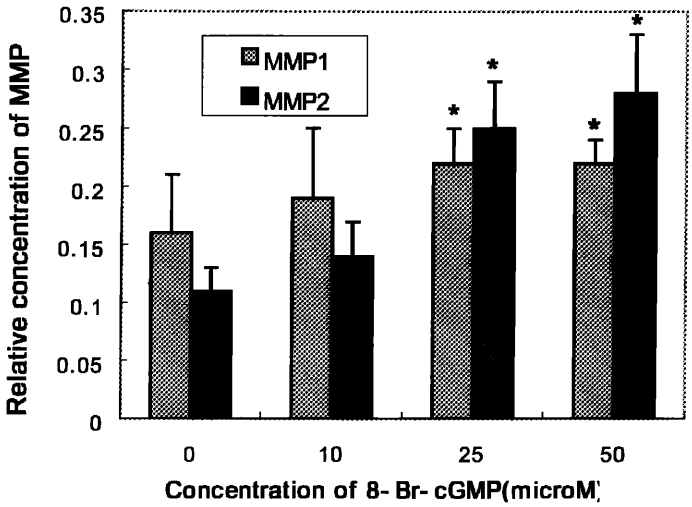

Figure 3. The effect of 8-Br-cGMP on the production of MMP-1 and MMP-2 by human dermal fibroblasts. *n = 3. $p < 0.05$ vs no treatment.

Graillet *et al.* (13), and they confirmed that NO plays an important role in the paracrine mediation of UV-induced melanogenesis. Figures 4 and 5 show the expression of inducible NOS (iNOS) and the production of NO from HDF treated with UV radiation of 30 J/cm^2. The mRNA of iNOS was increased by 15% when HDF was treated with UV of 30 J/cm^2 (Figure 4), and the production of NO was increased by 14% (Figure 5), but the results were not significant statistically ($p > 0.05$). Although the increase of iNOS and NO in the UV-irradiated HDF was not meaningful, the source of NO-influencing HDF is not negligible, considering the NO produced by keratinocytes. In a separate experiment, we confirmed that the addition of a culture supernatant of UV-irradiated keratinocytes to the culture medium of HDF also enhanced the production of MMP-1 and -2 by HDF (data not shown).

INHIBITION OF MMP PRODUCTION BY iNOS INHIBITORS

To confirm the action of NO on the MMP production by HDF, HDFs were treated with

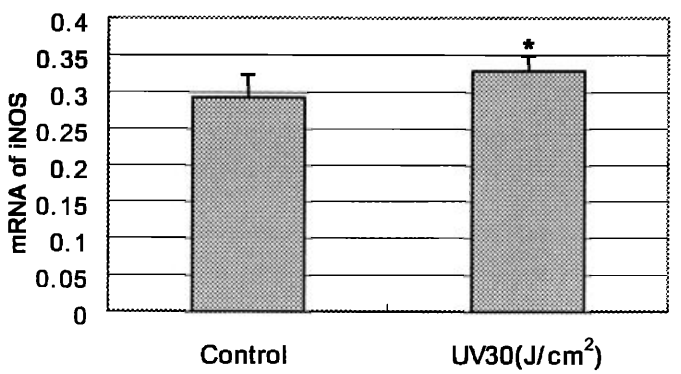

Figure 4. Production of inducible nitric oxide synthase by UV-irradiated human dermal fibroblasts. The intracellular mRNA of iNOS was determined by using the RT-PCR-ELISA method. *n = 4. $p > 0.05$ vs no UV exposure.

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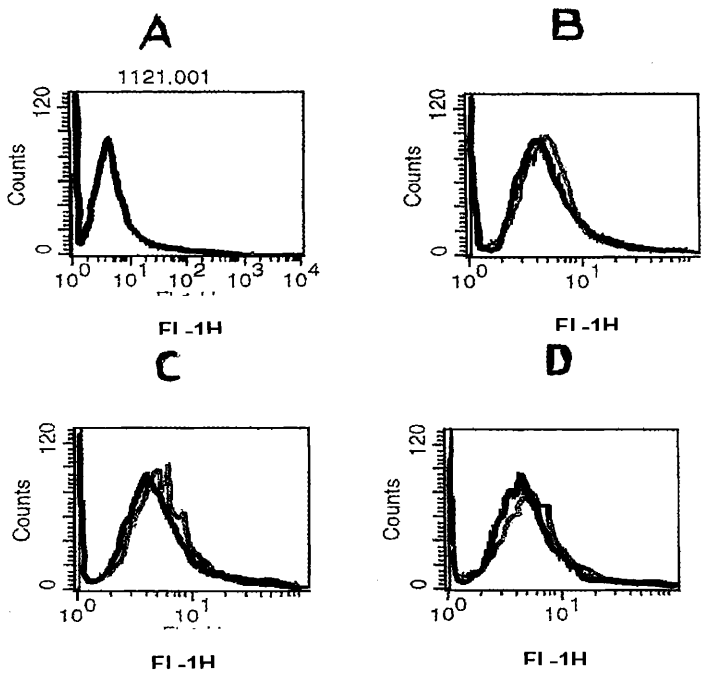

Figure 5. Production of nitric oxide by UV-irradiated human dermal fibroblasts. The intracellular NO was measured using NO sensor dye and fluorescence-activated cell sorter. Black line: without UV irradiation. White line: with UV irradiation. A: control. B: 10 J/cm^2. C: 20 J/cm^2. D: 30 J/cm^2.

various NOS inhibitors in the presence of UV irradiation (30 J/cm^2). AG, NAME, NAL, and BAC are NOS inhibitors that inhibit both the constitutive and inducible NOS. The extract of Scutellaria root is plentiful in various iNOS inhibitors including wogonin, baicalin, and baicalein (22). The enhancement of MMP production by UV irradiation was partially but significantly ($p < 0.05$) blocked by iNOS inhibitors such as AG and BAC, but not by NAME and NAL. Figure 6 shows that the enhanced production of MMP-1 or MMP-2 by UV irradiation was downregulated by the addition of AG and BAC. When 20 microM of BAC was added to the culture media, only 40% of MMP-1 and 42% of MMP-2 were produced, compared to the untreated case. These findings suggest that NO affects MMP-1 and -2 production through the cGMP pathway and is an important signal mediator in regulating the production of MMP by UV-irradiated HDF.

CONCLUSIONS

The effect of NO and iNOS inhibitors on the production of MMP-1 and -2 by UV-irradiated or non-irradiated HDF was studied. The addition of a NO donor, SNP, to the culture medium of HDF enhanced the production of MMP-1 and -2, while the addition of iNOS inhibitors such as AG and BAC downregulated the production of MMP-1 and -2 by UV-irradiated HDF. Although the production of NO by UV-irradiated HDF was not significant statistically, the influence of NO on HDF might not be negligible, considering the NO produced by UV-irradiated keratinocytes. From these results, we

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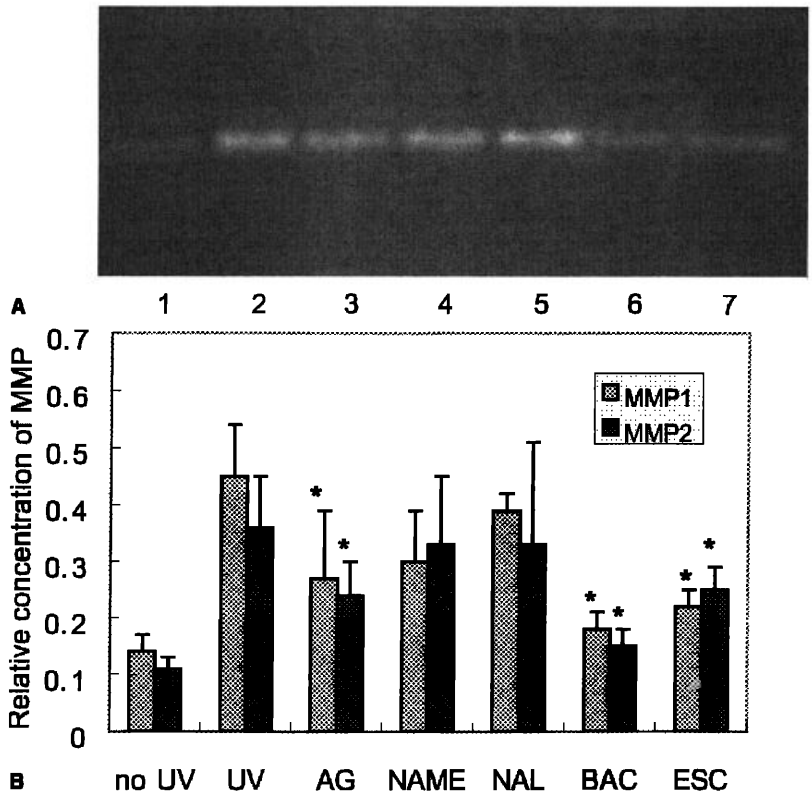

Figure 6. The effect of various NOS inhibitors on the production of MMP-1 and MMP-2 by UV-irradiated human dermal fibroblasts. (A) Zymography of MMP-2. 1. no UV. 2. UV (30 J/cm^2). 3. UV + AG (25 microM). 4. UV + NAME (50 microM). 5. UV + NAL (50 microM). 6. UV + BAC (20 microM). 7. UV + ESC (200 microg). (B) Proteins of MMP-1 and -2. AG: aminoguanidine. NAME: nitro-L-arginine methyl ester. NAL: nitro-L-arginine. BAC: baicalein. ESC: extract of Scutellaria root. *n = 4. $p < 0.05$ vs no treatment.

conclude that the production of MMP-1 and -2 by UV-irradiated HDF is regulated through the signaling pathway involving NO and can be downregulated using NOS inhibitors.

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