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Nitric Oxide Inhibits Ultraviolet B-induced Murine Keratinocyte Apoptosis by Regulating Apoptotic Signaling Cascades

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Cytotoxic effects of nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) are considered to be one of the major causes of inflammatory diseases. On the other hand, protective effects of NO on toxic insults-induced cellular damage/apoptosis have been demonstrated recently. Ultraviolet B (UVB)-induced apoptosis of epidermal keratinocytes leads to skin inflammation and photoageing. However, it has not been elucidated what kind of effects NO has on UVB-induced keratinocyte apoptosis. Thus, in the present study, we investigated the problem and demonstrated that NO from NO donor suppressed UVB-induced apoptosis of murine keratinocytes. In addition, NO significantly suppressed activities of caspase 3, caspase 8 and caspase 9 that had been upregulated by UVB radiation. NO also suppressed p53 expression that had been upregulated by UVB radiation and upregulated Bcl-2 expression that had been downregulated by UVB radiation. These findings suggested that NO might suppress UVB-induced keratinocyte apoptosis by regulating apoptotic signaling cascades in p53, Bcl-2, caspase3, caspase 8 and caspase 9.

Keywords: Nitric oxide (NO); Ultraviolet B (UVB); Cell damage; Keratinocytes; Apoptosis; Caspase

INTRODUCTION

Nitric oxide (NO), a reactive gaseous molecule with a simple structure and a short half-life, has multiple cellular functions such as vasodilatation, modulation of gene expression, signal transmission, cytotoxicity, apoptosis and cytoprotection.[1,2] When cells are stimulated with inflammatory cytokines such as interleukin-1β (IL-1β), interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) and/or lipopolysaccharide (LPS), large amounts of NO are synthesized via induction of inducible nitric oxide synthase (iNOS).[1,3–5] Increased synthesis of NO via iNOS causes cytotoxicity and is sometimes responsible for diseases such as rheumatoid arthritis, inflammatory bowel diseases, and insulin dependent diabetes mellitus.[6] Recently, it was demonstrated that cytotoxicity of IFN-γ and TNF-α for cultured murine vascular endothelial cells was mediated by NO synthesized via iNOS induction.[5] In contrast, rather cytoprotective effects of NO in some conditions were convincingly demonstrated.[7–9] For example, NO reportedly protected cells from hydrogen peroxide (H_2O_2)-induced cellular damage.[9–11] Thus, NO has both cytoprotective and cytotoxic effect, depending on its concentrations and/or unknown environmental factors.

Ultraviolet (UV) radiation, especially ultraviolet B (UVB) radiation, is one of the major environmental stimuli that induce damage/apoptosis of epidermal keratinocytes. After excessive UVB exposure, epidermal keratinocytes undergo apoptosis, which is characteristically recognized as sunburn cells. UVB radiation-induced keratinocyte apoptosis is playing an important role in preventing malignant transformation by removing cells with damaged DNA.[12,13] However, at the same time, excessive

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UVB radiation triggers erythema, lipid peroxidation in cellular membranes and cellular damage/apoptosis that may be responsible for many cutaneous disorders and photoageing.[14]

Recently, it was reported that the NO-mediated inflammatory response prevented UVB-induced lipid peroxidation[15] and that NO suppressed ultraviolet A (UVA)-induced apoptosis of endothelial cells.[16,17] However, effects of NO on UVB-induced damage/apoptosis of keratinocytes have remained unknown so far. Therefore, it appeared to be a very important problem to be solved. Thus in the present study, we investigated and elucidated that moderate doses of NO blocked UVB-induced keratinocytes apoptosis and that this inhibitory effect was closely linked to the suppression of several signal transduction molecules that lead to apoptosis.

MATERIALS AND METHODS

Materials

Pam 212 murine keratinocytes originally established by Yuspa *et al.*[18] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin G; 100 U/ml, and streptomycin; 100 μg/ml) at 37°C, under 5% CO_2 in air. A slow reacting NO donor NOC 18 was purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

UV Radiation

The UVB source was a bank of fluorescent sun lamps (FL20SE, Toshiba Light and Technology Co., Tokyo, Japan), which emit UV radiation at wavelengths ranging from 280 to 320 nm, with a peak wavelength of 312.5 nm. The average flux intensity measured with an UVR-305/365D digital radiometer (Tokyo Kogaku Kikai K.K., Tokyo, Japan) was 0.37 mW/cm^2 at the level of the cell surface. Pam 212 keratinocytes were grown in 60 mm dishes in culture medium (DMEM supplemented with 10% FBS and antibiotics) for 24 h, then the medium was removed and the cells were washed twice with phosphate buffered saline (PBS) and overlaid with 0.5 ml PBS. Cells were then irradiated with various doses of UVB, after which PBS was replaced by culture medium, to which various doses of NOC 18 (the NO donor) were added. The cells were then incubated for designated periods at 37°C under 5% CO_2 in air.

Quantification of Apoptotic Cells

Two methods were used to quantify the apoptotic response after UVB radiation. First, morphological changes of cell nuclei were observed with Giemsa's stain. At designated time after the stimulation, cells were collected with trypsinization, fixed with methyl alcohol mixed with acetic acid and stained with Giemsa's solution. The rate of apoptotic cells characterized by fragmented nuclei and/or chromatin condensation was calculated by counting more than 300 cells per sample as described previously.[5,19] Secondly, to further confirm UVB-induced apoptotic response, a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL)-based apoptotic detection kit: ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Serologicals Corps, Spalding Drive Norcross, GA, USA) was used. By using this kit, apoptotic cells were detected *in situ* by the indirect TUNEL method, utilizing an anti-digoxigenin antibody conjugated with FITC. The rate of apoptotic cells were calculated by counting more than 1000 cells per sample by using fluorescence microscopy.

Determination of Caspase Activities

At designated time after stimulation with UVB and/or the NO donor NOC 18, cell extracts were prepared and their caspase activities were determined using the CaspAce™ colorimetric assay system (Promega, Madison, WI, USA), the FLICE/Caspase-8 colorimetric protease assay kit (Medical & Biological Laboratories Co., Nagoya, Japan) and the Caspase-9/Mch6 colorimetric protease assay kit (Medical & Biological Laboratories Co., Nagoya, Japan). Briefly, after cell extracts were prepared by freezing and thawing according to the manufacturer's instructions, caspase activities in the extracts were measured as follows. Cell extracts were reacted with caspase substrates conjugated with p-nitroaniline (pNA): Ac-DEVD-pNA, IETD-pNA and LEHD-pNA for caspase 3, caspase 8 and caspase 9, respectively. After incubating for 4 h at 37°C, the absorbance at 405 nm was measured using a microtiter plate reader (Microplate Reader, Model 3550, Bio-Rad, Tokyo, Japan).

Analysis of p53 and Bcl-2 Expressions by Western Blotting

Thirty hours after stimulation, cells were scraped off from the dishes, suspended in PBS and disrupted by sonication on ice. After centrifugation at 12000 rpm for 10 min at 4°C, the supernatants were collected and were used as samples. Protein concentrations of samples were evaluated by the Bradford dye-binding assay using a protein assay kit (Bio-Rad, Tokyo, Japan). Sample proteins were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and were then electrotransferred from the gel to a polyvinylidene difluoride (PVDF)

membrane (Hybond-P, Amersham Biosciences, Buckinghamshire, England). After blocking with 0.3% skimmed milk in PBS overnight at 4°C, the membranes were immunoreacted with a rabbit anti p53 antibody or a rabbit anti Bcl-2 antibody (Santa Cruz, CA, USA) for 1 h at room temperature. The membranes were washed with washing buffer (0.01 M PBS with 0.1% Tween 20) and reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase (Dako, Tokyo, Japan) contained in 0.3% skimmed milk for 1 h at room temperature. The membranes were then washed with washing buffer for 1 h and bound peroxidase activity was visualized using Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Densitometric analyses were performed from three independent Western blot reaction products by using an optometric scanner (GT-6000, Epson) and the NIH image version 1.63 software.

RESULTS

To confirm and characterize the apoptotic response after UVB radiation, we used three methods: (1) Giemsa's stain to observe the morphological changes of nuclei, (2) a TUNEL-based *In Situ* Apoptosis Detection Kit to detect DNA fragmentation, and (3) measurement of caspase activity. As shown in Fig. 1A, Giemsa's stain demonstrated

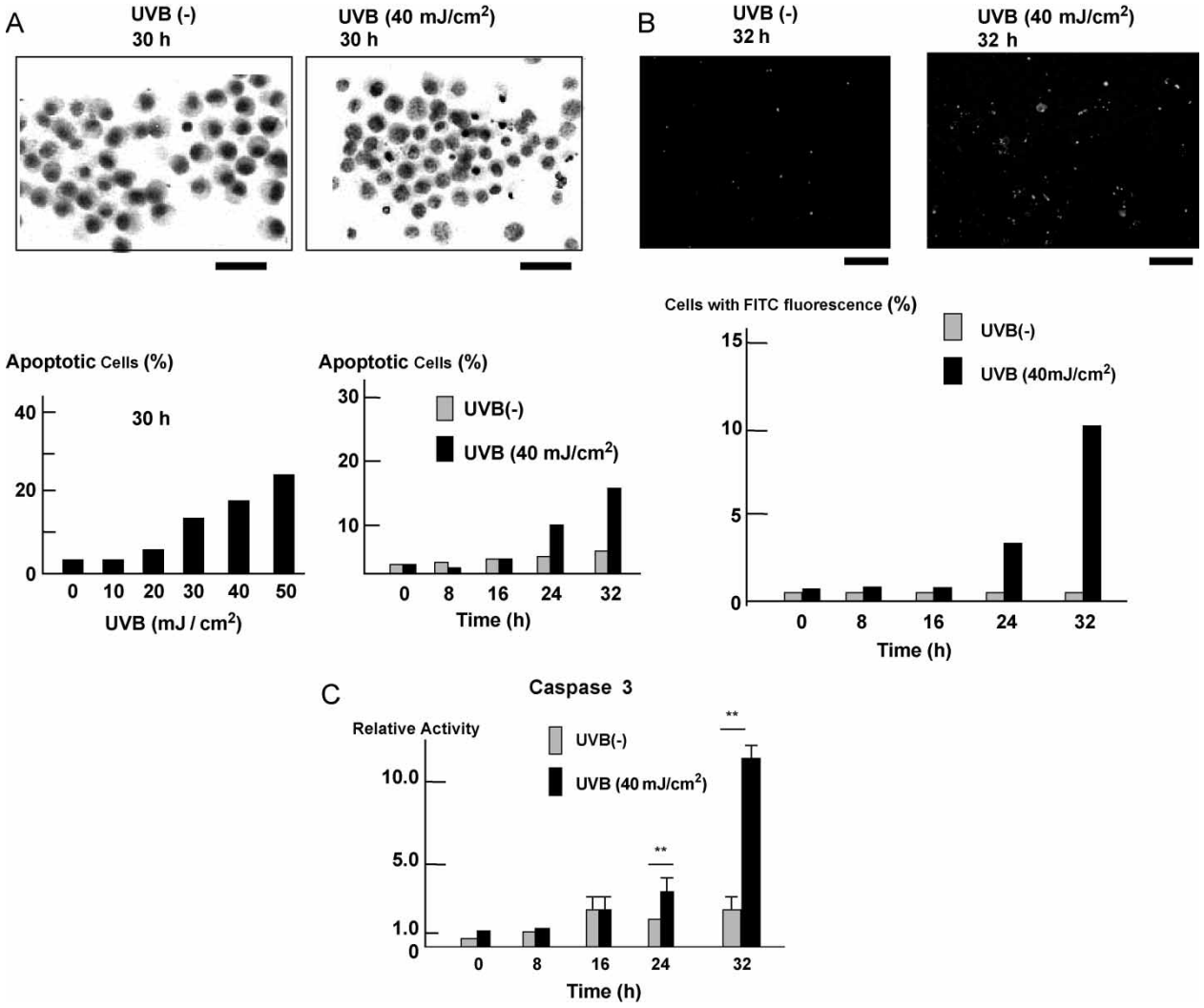

FIGURE 1 Effect of UVB radiation on keratinocyte apoptosis. Pam 212 murine keratinocytes grown in DMEM supplemented with 10% FBS were stimulated with UVB radiation (0–50 mJ/cm^2). After incubating for 30 h, cells were collected with trypsinization and stained with Giemsa's solution (A). Scale bars: 50 μm. The percentages of cells with fragmented and/or condensed nuclei were calculated by counting more than 300 cells per sample. The UVB dose-dependent apoptotic changes were demonstrated in the lower left and the time course of apoptotic changes were demonstrated in the lower right (A). To further confirm the apoptotic response, analyses using a TUNEL-based apoptotic detection kit were also performed as described in "Materials and methods" section. Thirty-two hours after UVB radiation, apoptotic cells were detected with this kit and observed by fluorescence microscopy (B). Scale bars: 100 μm. Also the time course analyses of apoptotic response were performed with this kit. The rate of cells with FITC fluorescence was calculated by counting more than 1000 cells per sample (B, low). The caspase 3 activity was detected at designated time after UVB radiation (0, 40 mJ/cm^2) as described in "Materials and methods" section (C). Each data point represents the mean ± standard deviation (SD) ($n = 3$). **: $p < 0.01$ in Students' t test.

that the percentage of cells with significant nuclear fragmentation and/or chromatin condensation increased in proportion to the UVB doses and that more than 30 mJ/cm^2 UVB radiation caused these changes. In addition, according to the time course analyses, these nuclear changes appeared at 24 h after UVB radiation and were significantly observed at 32 h (Fig. 1A). Analyses with a TUNEL-based *In Situ* Apoptosis Detection Kit demonstrated that 40 mJ/cm^2 UVB radiation induced significant DNA fragmentation (Fig. 1B) and that these changes began to appear at about 24 h after UVB radiation and were most remarkably observed at 32 h (Fig. 1B). The time course analyses of caspase 3 activity demonstrated that it began to rise at 24 h after UVB radiation and significantly increased at 32 h. Thus, UVB-induced apoptosis was characterized and the time course of each apoptotic response was made clear through these assays.

Recently, it was reported that NO blocked endothelial cell apoptosis induced by UVA.[16,17] Therefore, it was an intriguing question whether NO also blocked UVB-induced apoptosis of keratinocytes or not. Thus, we examined the effect of NO on UVB-induced keratinocyte apoptosis. According to the manufacturer's data, the slow releasing NO donor NOC 18 has a half-life of 21 h and that from 500 μmol NOC 18, the total amount of 500 μmol NO is released into the medium at 37°C in 0.1 M phosphate buffer (pH 7.4) within the first 21 h.[20] We confirmed the dose-dependent production of NO from NOC 18 after the addition of NOC 18 by measuring nitrite and nitrate (end products of NO) accumulated in culture medium (data not shown).[5] In Fig. 2A, it is demonstrated that stimulation with UVB radiation (40 mJ/cm^2) alone or with NOC 18 (1000 μM) alone caused remarkable nuclear fragmentation and/or chromatin condensation as

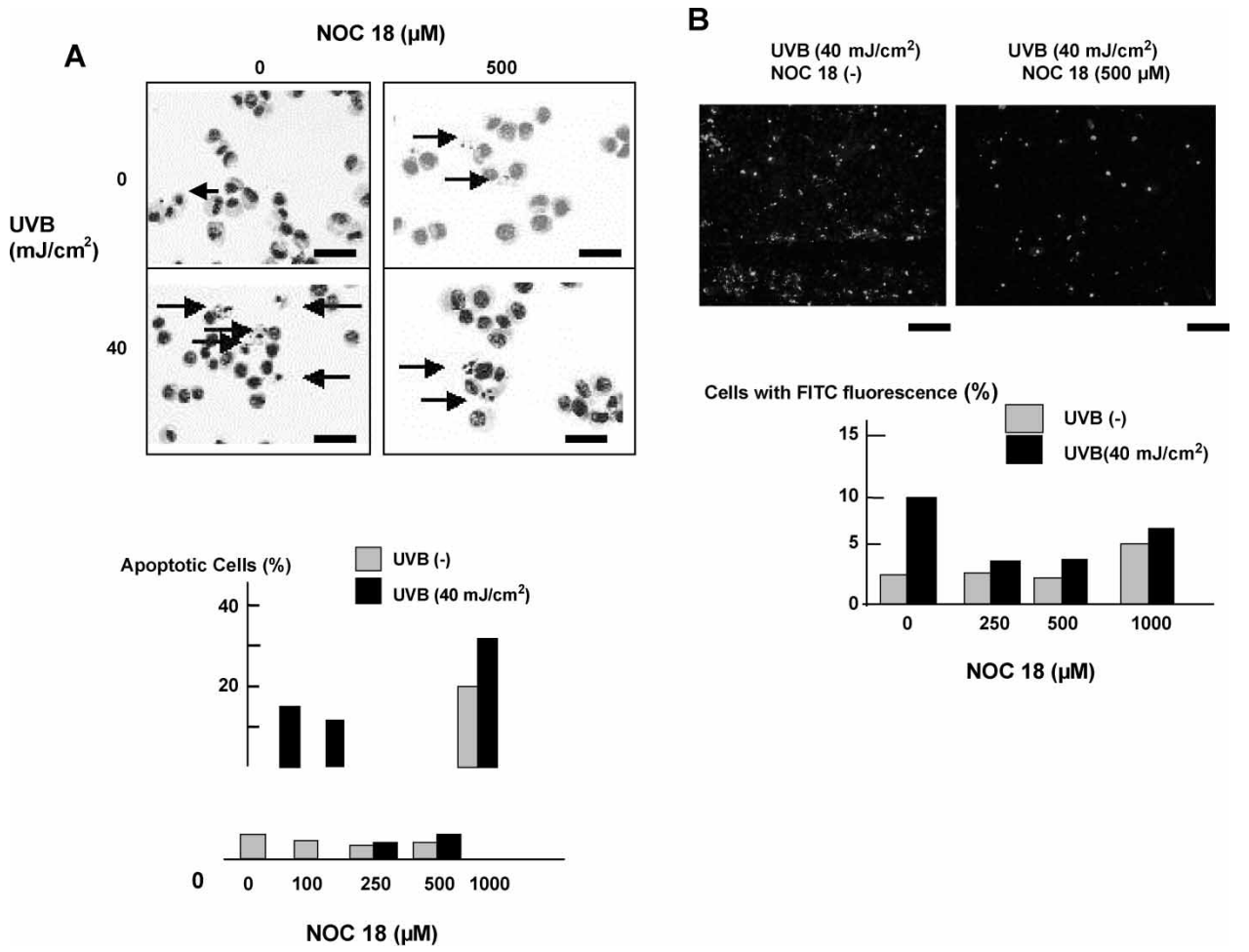

FIGURE 2 Effect of NO on UVB-induced keratinocyte apoptosis. Pam 212 keratinocytes were stimulated with UVB radiation (0, 40 mJ/cm^2). Soon after UVB radiation, various doses of a slow releasing NO donor NOC 18 (0, 100, 250, 500 and 1000 μM) were added to the medium. After incubating for 30 h, cells were collected and stained with Giemsa's solution (A). Scale bars: 50 μm. The percentages of cells with fragmented and/or condensed nuclei (arrow) were calculated by counting more than 300 cells per sample (A). To further confirm the apoptotic response, analyses with a TUNEL-based apoptotic detection kit were performed as described in "Materials and methods" section. Thirty-two hours after UVB radiation, cells were harvested and apoptotic cells were detected with this method. Cells with FITC fluorescence were observed by using fluorescent microscopy (B). Scale bars: 100 μm. The rate of cells with FITC fluorescence was calculated by counting more than 1000 cells per sample (B, low).

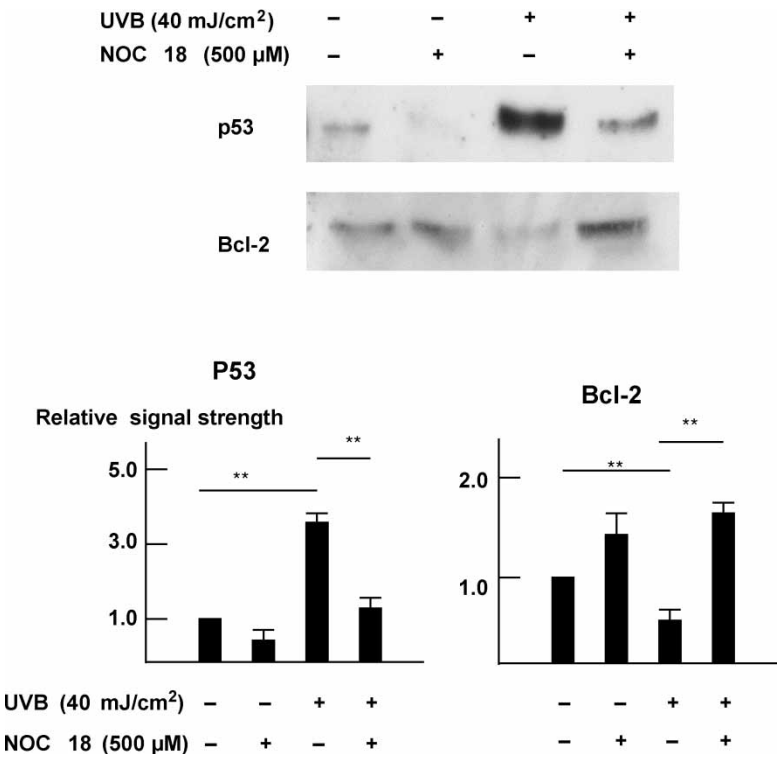

FIGURE 3 Effect of NO on p53 and Bcl-2 protein expressions. Pam 212 keratinocytes were stimulated with UVB radiation (0, 40 mJ/cm^2). Soon after UVB radiation, the NO donor NOC 18 (0, 500 μM) was added to the medium. After incubating for 30 h, cells were harvested and the protein expressions of p53 and Bcl-2 were detected by Western blotting as described in "Materials and methods" section. Densitometric analyses were performed from 3 independent Western blot reaction products by using an optometric scanner (GT-6000, Epson) and the NIH image version 1.63 software. Each data point represents the mean ± standard deviation (SD) ($n = 3$). **: $p < 0.01$ in Students' t test.

detected by Giemsa's stain. However, lower doses of NOC 18 (250–500 μM) significantly suppressed apoptotic nuclear changes induced by UVB radiation (40 mJ/cm^2) (Fig. 2A). Similarly, analyses with a TUNEL-based *In Situ* Apoptosis Detection Kit demonstrated that the addition of lower doses of NOC 18 (250–500 μM) suppressed UVB-induced apoptosis significantly (Fig. 2B). These results suggested that lower doses of NOC 18 blocked UVB-induced keratinocyte apoptosis.

Next, we investigated the site of inhibition by NO in UVB-induced apoptotic signal transduction. Signal transduction pathways to apoptosis triggered by UVB radiation are generally classified into two pathways; one pathway involving the regulation of p53, Bax, and Bcl-2 leading to the release of cytochrome *c* from mitochondria, and another pathway through the activation of Fas leading to the activation of caspase 8 and caspase 3[12,13] (Fig. 5). To clarify pathways through which NO inhibits UVB-induced apoptosis, we first examined protein expressions of p53 and Bcl-2 by Western blotting analyses. As shown in Fig. 3, the expression of p53 increased after UVB radiation (40 mJ/cm^2), and the increased expression was suppressed by the addition of NOC 18 (500 μM). In contrast, the expression of the anti-apoptotic protein Bcl-2 was suppressed by UVB radiation and was upregulated by the addition of NOC 18 (500 μM). Thus, lower concentrations of NOC 18 inhibited UVB-induced apoptosis probably by suppressing the pathway through p53 and Bcl-2. Next, we examined the effect of UVB radiation and NO on activities of caspase 3, caspase 8 and caspase 9. As shown in Figs. 4A–C, activities of caspases increased after UVB radiation (40 mJ/cm^2), and the increased activities were significantly suppressed by the addition of NOC 18 (500 μM). These results suggested that the inhibition of UVB-induced keratinocyte apoptosis by NO might be due to the modulation of apoptotic signaling cascades in p53, Bcl-2, caspase3, caspase 8 and caspase 9.

DISCUSSION

Recently, it was reported that NO protected endothelial cells and fibroblasts from injuries induced by toxic insults such as reactive oxygen species.[9–11,16,17,21] In the present study, we examined whether NO protected UVB-induced keratinocyte apoptosis or not. As a result, lower doses of NOC 18 protected keratinocytes from UVB-induced apoptosis, while high doses of NOC 18 had cytotoxic effects.

Next, the target sites of inhibition by NO in UVB-induced apoptosis were investigated. As the molecular mechanisms of UVB-induced keratinocyte

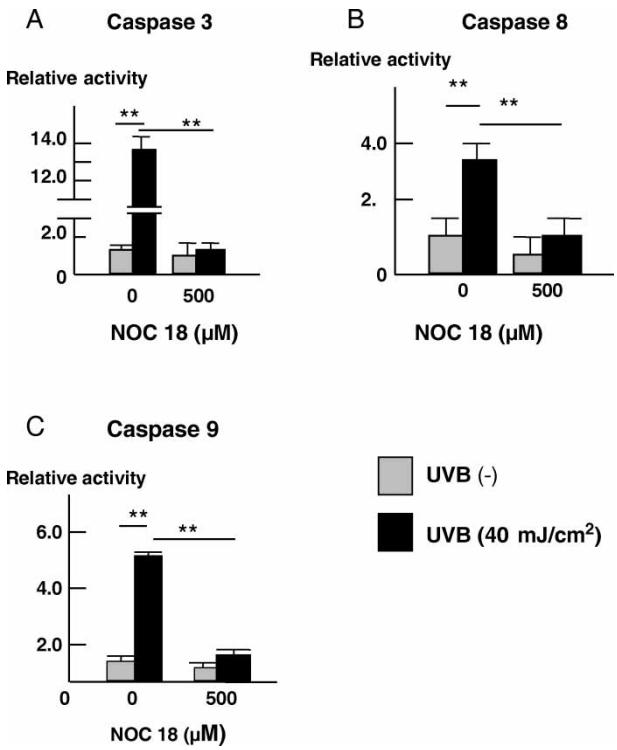

FIGURE 4 Effect of NO on caspase activities. Pam 212 keratinocytes were stimulated with UVB radiation (0, 40 mJ/cm^2). Soon after UVB radiation, the NO donor NOC 18 (0, 500 μM) was added to the medium. After incubating for 30 h, cells were harvested and cell extracts were prepared and activities of caspase 3 (A), caspase 8 (B) and caspase 9 (C) were determined by measuring the absorbance at 405 nm as described in "Materials and methods" section. Relative activities were shown in the figure. Each data point represents the mean ± SD ($n = 3$). **: $p < 0.01$ in Students' t test.

apoptosis, two signaling pathways have been considered. The first pathway is mediated by p53, Bcl-2, cytochrome *c* release from mitochondria and the activation of caspase 9 and caspase 3, as shown in Fig. 5.[12,13] And the second pathway is mediated by surface Fas expression, FADD activation, and the activation of caspase 8 and caspase 3. The present findings revealed that the expression of p53 was upregulated by UVB radiation, and was suppressed by the addition of NO. In contrast, the expression of Bcl-2 was suppressed by UVB radiation, and was upregulated by the addition of NO. Activities of downstream caspases such as caspase 9 and caspase 3 were markedly increased after UVB radiation and were recovered to the basal level by the addition of NO. These findings suggested that the inhibition of the first signaling pathway that mediated p53, Bcl-2, caspase 9 and caspase 3 might, at least in part, account for the protective effect of NO on UVB-induced apoptosis. In association with the present results, it was reported that inhibitory effect of NO on UVA-induced apoptosis of endothelial cells was closely linked with the downregulation of Bax and the upregulation of Bcl-2.[16] Moreover, NO reportedly prevented apoptosis of splenic B lymphocytes or hepatocytes by sustaining Bcl-2 levels.[22,23] Thus, it was suggested that the modulation of the first pathway by NO might be at least one of the key events in the inhibition of apoptosis by NO.

In addition, we demonstrated that the activities of caspase 8 and caspase 3 were markedly upregulated by UVB radiation and were recovered to the basal level

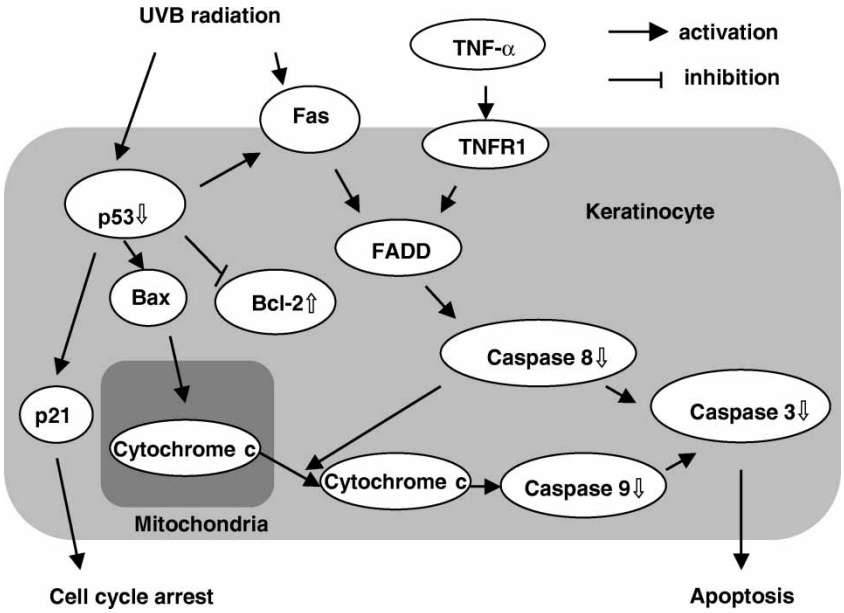

FIGURE 5 Effects of NO on signaling pathways to UVB-induced keratinocyte apoptosis. Arrows in the figure (⇧ and ⇩) indicate the effect of NO on signal transduction molecules. ⇧: upegulation by NO, ⇩: downregulation by NO.

by the addition of NO. In association with the present results, it was demonstrated that NO inhibited activities of caspase family members through S-nitrosylation of conserved cysteine in the active site of caspases.[24–26] Among caspases, the suppression of caspase 3 was considered to be essential for the inhibition of apoptosis by NO.[27,28] Thus, it was suggested that the inhibition of the second signaling pathway that mediated the activation of caspase 8 and caspase 3 might, at least in part, account for the protective effect of NO on UVB-induced apoptosis. The inhibition of UVB-induced apoptosis by NO might be due to the inhibition of several sites in the apoptotic signaling cascades as shown in Fig. 5.

Thus, it was elucidated that UVB-induced keratinocyte apoptosis might be suppressed, when appropriate concentrations of NO were present. For example, when cells are stimulated with cytokines such as IFN-γ, TNF-α and /or IL1-β, large amounts of NO are produced via iNOS induction.[3–5] Also, UVB radiation to the skin might induce iNOS expression as demonstrated in *in vivo* study.[29] Moreover, when NO donors are applied exogenously for therapeutic purposes, large amounts of NO may be present in the epidermis. In these situations, considerable amounts of NO may be present in the epidermis, enabling the suppression of UVB-induced keratinocyte apoptosis. The slow releasing NO donor NOC 18 that we used in the present study has a half-life of 21 h. From 500 μmol NOC 18, the total amount of 500 μmol NO is released into the medium at 37°C in 0.1 M phosphate buffer (pH 7.4) within the first 21 h (average release: 0.40 μmol/min).[20] Considering that the basal concentration of NO *in vivo* is approximately 1–5 μM[30–32] and that stimulation with cytokines induces NO production (sometimes by more than 10- to 100-folds) via iNOS induction,[26,33,34] it seems possible that sufficient doses of NO might be physiologically present, playing protective roles, especially under inflammatory conditions.

In conclusion, we demonstrated in the present study that lower doses of NOC 18 inhibited UVB-induced apoptosis of keratinocytes. In addition, we investigated the mechanisms and demonstrated that NO modulated apoptotic signaling molecules such as p53, Bcl-2, caspase 3, caspase 8 and caspase 9. These findings suggested that NO might suppress UVB-induced keratinocyte apoptosis by regulating apoptotic signaling cascades in p53, Bcl-2, caspase 3, caspase 8 and caspase 9. To clarify more precise mechanisms underlying UVB-induced keratinocyte apoptosis, further investigations are required.

Acknowledgements

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