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UVB light suppresses nitric oxide production by murine keratinocytes and macrophages

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Abstract

Nitric oxide is an important mediator of excessive cell growth and inflammation associated with many epidermal proliferative disorders. It is a highly reactive oxidant generated in keratinocytes and macrophages via the inducible form of the enzyme nitric oxide synthase (NOS2). In the present studies, we examined the effects of ultraviolet light (UVB, 2.5–25 mJ/cm^2) on interferon-γ (IFN-γ)-induced expression of NOS2 in these cells. Transient transfection assays using wild-type and mutant NOS2 promoter/luciferase reporter constructs showed that DNA binding of the transcription factors Stat1 and NF-κB was essential for optimal expression of the *NOS2* gene. Whereas NF-κB was constitutively expressed in both cell types, Stat1 phosphorylation and nuclear binding activity were dependent upon IFN-γ. UVB light, which is used therapeutically to treat inflammatory dermatosis, was found to suppress IFN-γ-induced expression of NOS2 mRNA and protein, and nitric oxide production in both keratinocytes and macrophages. In macrophages, this was associated with complete inhibition of NF-κB nuclear binding activity and partial (∼20–25%) reduction of Stat1 activity. In keratinocytes, both responses were partially reduced at the highest doses of UVB light (15–25 mJ/cm^2). Whereas in macrophages UVB light suppressed NOS2 wild-type promoter-luciferase reporter activity, this activity was stimulated in keratinocytes. These data suggest that UVB light functions to suppress *NOS2* gene expression in macrophages by inhibiting the activity of key regulatory transcription factors. In contrast, in keratinocytes, inhibition occurs downstream of *NOS2* promoter activity.

Keywords: Nitric oxide; Ultraviolet light; Interferon-γ; Psoriasis; Skin

1. Introduction

Ultraviolet light of high energy and shorter wavelengths (UVB, 290–320 nm) is effective in photochemotherapy for psoriasis and atopic dermatitis. The mechanisms underlying the therapeutic actions of UVB light are unknown [1]. Whereas in normal skin, UVB light can induce an inflammatory response, it exhibits anti-inflammatory and immunosuppressive activity in diseased tissue, which may contribute to its therapeutic efficacy. Many studies have implicated nitric oxide as a key cytotoxic and proinflammatory mediator in the skin [2,3]. It is generated in keratinocytes [4], macrophages [5], dermal fibroblasts [6], dermal microvascular endothelial cells [7], and melanocytes [8] in response to inflammatory cytokines via a high capacity NOS2. Increased NOS2 expression has been observed in many inflammatory skin diseases including psoriatic lesions, atopic dermatitis, and contact dermatitis [9–11]. Moreover, nitric oxide synthase inhibitors have been used to reverse these conditions with varying degrees of success [3,12,13].

Inflammatory cytokines present in injured or diseased skin include IFN-γ, tumor necrosis factor-α, interleukin-1β, and interleukin-6 [14–16]. Our laboratories have

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Abbreviations: AP-1, activator protein-1; BSO, buthionine sulfoximine; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate; DMEM, Dulbecco's modified Eagle's medium; GAS, IFN-γ activated sequence; GSH, glutathione; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NF-κB, nuclear factor-κB; NOS2, inducible form of nitric oxide synthase; RT-PCR, reverse transcriptase-polymerase chain reaction; Stat1, signal transducer and activator of transcription-1; TdR, thymidine.

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demonstrated previously that IFN-γ readily induces NOS2 and nitric oxide production in keratinocytes and macrophages, and this correlated inversely with cellular proliferation [4,17]. These findings suggest that nitric oxide is potentially important in the regulation of wound healing [3,4]. Treatment of mice with UVB light has been reported to suppress nitric oxide production by peritoneal macrophages stimulated with IFN-γ or tumor necrosis factor-α *in vitro* [18]. This is thought to be due to UVB light-induced immune suppression [18,19]. The present studies show that therapeutic doses of UVB light are also effective inhibitors of nitric oxide production as well as NOS2 expression in keratinocytes and macrophages *in vitro*. In macrophages, this appears to be due to inhibition of transcription factors regulating the expression of the *NOS2* gene, while in keratinocytes, events downstream of this process are likely to be important in regulating expression of the gene product. Inhibition of nitric oxide production in the skin may be an important mechanism contributing to the therapeutic actions of UVB light in epidermal proliferative disorders.

2. Materials and methods

2.1. Reagents

[γ-^{32}P]ATP (111 TBq/mmol), [*methyl*-^{3}H]TdR (740 GBq/mmol), and [α-^{32}P]CTP (111 TBq/mmol) were obtained from NEN Life Science Products. Recombinant mouse IFN-γ was purchased from Life Technologies. LPS derived from *Salmonella enteritidis* and all other chemicals were obtained from the Sigma Chemical Co. Protein was quantified using the detergent compatible (Dc) protein assay (Bio-Rad) with BSA as the standard. Antibodies against NOS2, p50, p65, AP-1 and all secondary antibodies were purchased from Santa Cruz Biotechnology. Antibodies to phospho-Stat1 and Stat1 were from Transduction Laboratories, and to ERK1/2, phospho-ERK1/2, JNK/SAPK, phospho-JNK/SAPK, p38 and phospho-p38 MAP kinases from Cell Signaling Technology.

2.2. Cells and treatments

RAW 264.7 mouse macrophages and PAM 212 mouse keratinocytes were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum as described previously [17,20]. Cells (2×10^5) were plated into 6-well culture dishes in growth medium. At 80% confluence, the medium was replaced with phenol red-free DMEM lacking serum. The tops of the culture dishes were then removed, and the cells were exposed to UVB light from a bank of 2 FS40BL light bulbs at a distance of approximately 25 cm above the culture dishes. UVB lights were calibrated using an International Light IL-1700 UV-radiometer. In some experiments, a filter was used to eliminate wavelengths below 290 nm (UVC light); this did not block the inhibitory effects of UVB light. Cell viability was assayed by trypan blue dye exclusion, an MTS assay kit [21], or by [^{3}H]TdR uptake. For uptake studies, cells were plated in 24-well culture dishes. Following UVB light treatment, the culture medium was removed, and the cells were refed with phenol red-free DMEM supplemented with IFN-γ (100 U/mL) and [^{3}H]TdR (2 μCi/mL, final concentration). After 3 hr at 37°, the labeling medium was decanted, and the cells were washed (3 × 1 mL) with ice-cold PBS and then precipitated with 1 mL of ice-cold 5% trichloroacetic acid (TCA) for 10 min. The pellets were then washed with TCA (3 × 1 mL), solubilized in 0.25 mL of 0.2 N NaOH, and assayed for radioactivity.

To deplete intracellular GSH, cells were treated with BSO (100 μM, 6 hr). This treatment reduced intracellular GSH from 155 ± 7 to 29 ± 2 nmol/10^6 cells (mean ± SEM, $N = 3$) in macrophages, and from 25 ± 2 to 5 ± 1 nmol/10^6 cells in keratinocytes, as measured using a 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay [22]. Hydroperoxide formation in cells was measured using DCFH-DA in conjunction with flow cytometry as previously described [4]. Nitric oxide production was quantified by the accumulation of nitrite in the cell culture medium using the Greiss reagent with sodium nitrite as the standard [4,17].

2.3. Protein immunoblots

Extracts containing 20 μg of protein were electrophoresed on 7.5 or 10% polyacrylamide gels and then were transferred onto nitrocellulose membranes as previously described [17]. After blocking with 5% non-fat dried milk in TTBS buffer (Tris-buffered saline with 0.1% Tween 20) for 1 hr, membranes were incubated with primary antibodies overnight at 4° followed by horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. Antigen–antibody complexes were visualized on the blots using enhanced chemiluminescence (ECL) reagents (Perkin-Elmer).

2.4. RNA isolation and RT-PCR

RNA was isolated from the cells using TRIzol reagent (GIBCO BRL) following the instructions of the manufacturer. First strand cDNA synthesis was performed using SUPERSCRIPT II for RT-PCR (GIBCO BRL) with 0.4 μg RNA in each reaction. PCR reactions utilized 1 μL of cDNA, mouse NOS2 upstream primer 5′-GCTCATCCGGTACGCTGCTA-3′, downstream primer 5′-TCCAATCTCGGTGCCCATGTA-3′ (Ambion Gene-Specific Relative RT-PCR kit), and *Taq* DNA polymerase (GIBCO BRL). The PCR amplification protocol was a modification of the protocol of the manufacturer to allow for NOS2 quantitation using 18S as an internal standard. Varying cycles were used to optimize quantitation. The PCR products were

amplified using a 1-min hot start at 95° and 25 cycles of 30 s at 95°, 30 s at 56°, and 60 s at 72° followed by a final extension at 72° for 10 min. Products were separated by electrophoresis on an 8 M urea, 6% polyacrylamide gel, followed by autoradiography. Data were analyzed using the Eagle Eye II digital documentation system (Stratagene).

2.5. Transient transfections

Reporter plasmids in which the expression of firefly luciferase is driven by wild-type or mutated promoter constructs of the murine inducible *NOS2* gene [23,24] were used to determine which transcription factors were necessary for NOS2 expression after cells were treated with UVB light and IFN-γ. Mutated promoter constructs for both of the NF-κB binding sites (κBI_m and κBII_m), the GAS element (GAS_m), the ISRE element ($ISRE_m$), deletion of the entire basal promoter (H/Bx), deletion of the entire enhancer promoter (Bx/H), and deletion of all the upstream regulatory elements leaving only the TATA box and the transcriptional start site (P/H) were used [23,24]. To assess transfection efficiency, cells were co-transfected with a *Renilla* luciferase plasmid (pRL-TK, Promega). Plasmids were introduced into RAW 264.7 cells by electroporation using a Bio-Rad Gene Pulser II system (200 V, 1000 μF, 0.4 mm cuvettes) and then seeded into 24-well culture plates (2×10^5 cells/well). PAM 212 cells were transfected using Superfect reagent (Qiagen Inc.). The NOS2 constructs and the *Renilla* luciferase construct were used at a 10:1 molar ratio. Transfected cells were incubated for 24 hr at 37° prior to treatment with UVB light and/or IFN-γ (100 U/mL) and LPS (1 μg/mL). After 6 hr, luciferase activities were assessed using the Dual Luciferase Assay (Promega) with a Turner model 20/20 luminometer. Results are reported as the ratio of firefly to *Renilla* luciferase activity in the extract.

2.6. Electrophoretic mobility shift assays

Briefly, to prepare nuclear extracts, cells were scraped from the culture dishes into 1 mL of PBS and centrifuged (400 *g*, 10 min, 4°). The pellets were resuspended in 400 μL of hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 10 μg/mL of pepstatin, 2 μg/mL of aprotinin, 1 mM dithiothreitol (DTT), 2 μg/mL of 1-chloro-3-tosylamido-7-amino-2-heptanone, 5 mM NaF, and 10 mM sodium molybdate] and allowed to swell on ice for 15 min. Nonidet P-40 (NP-40) was then added to a final concentration of 0.5%. After rapid mixing for 15 s, the nuclei were pelleted (4000 *g*, 1 min, 4°), and the supernatants containing cytosolic proteins were collected. Each pellet was resuspended in 100 μL of nuclear extract buffer B (hypotonic buffer A supplemented with 20% glycerol and 0.4 M KCl) and shaken vigorously at 4° for 30 min. Nuclear extracts, obtained from the supernatants after centrifugation (13,000 *g*, 15 min, 4°), were stored at −70°. Electrophoretic mobility shift assays were performed using the method of Yamamoto *et al*. [25]. Nuclear extracts (20 μg) were incubated with 1 ng of a ^{32}P-labeled GAS probe (sense, 5′-GATCGATTTCCCCGAAT-3′; antisense, 5′-CATGATTTCGGGGAATC-3′) or a ^{32}P-labeled NF-κB probe (sense, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; antisense, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) or a ^{32}P-labeled AP-1 probe (sense, 5′-GATCCTTCGTGACTCAGCGGGATCCTTCGTGACTCAGCGG-3′; antisense, 5′-GATCCCGCTGAGTCACGAAGGATCCCGCTGAGTCACGAAG-3′) at room temperature for 15 min in a reaction mixture containing 30 μg BSA and 3 μg poly(dI/dC) in binding buffer containing 7.5% glycerol, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 35 mM NaCl, and 7.5 mM HEPES (pH 8) in a total volume of 25 μL. The DNA–protein complex was separated on 4.5% native polyacrylamide gels. Binding specificity was determined by competition using a 100-fold excess of unlabeled oligonucleotide. For supershift analysis, nuclear extracts were preincubated with 1 μg of appropriate antibodies prior to their addition to the reaction mixture. Radioactive bands were detected by autoradiography.

3. Results

3.1. Suppression of nitric oxide production by UVB light

In murine keratinocytes and macrophages, IFN-γ readily stimulated the production of nitric oxide (Fig. 1). UVB light caused a dose-dependent suppression of IFN-γ-induced nitric oxide production in both cell types (Fig. 1A). This was associated with the inhibition of IFN-γ-induced expression of NOS2 protein and mRNA (Fig. 1B and C). In the absence of IFN-γ, UVB light had no effect on NOS2 protein or mRNA expression, and it did not induce nitric oxide production by the cells (data not shown). The effects of UVB light were not due to cytotoxicity since the cells remained viable, as determined by MTS and trypan blue viability assays (data not shown). Moreover, [^{3}H]TdR uptake studies showed that UVB light treatment had no effect on cellular proliferation (Fig. 2A). Both keratinocytes and macrophages recovered from the inhibitory effects of UVB light on NOS2. Whereas in keratinocytes, recovery was complete within 12 hr, in macrophages this response was delayed for up to 24 hr (Fig. 2B and C).

In both keratinocytes and macrophages, UVB light treatment resulted in an increase in levels of intracellular reactive oxygen intermediates as measured with the hydroperoxide-sensitive dye DCFH; greater amounts were produced in macrophages than keratinocytes (Fig. 3A).

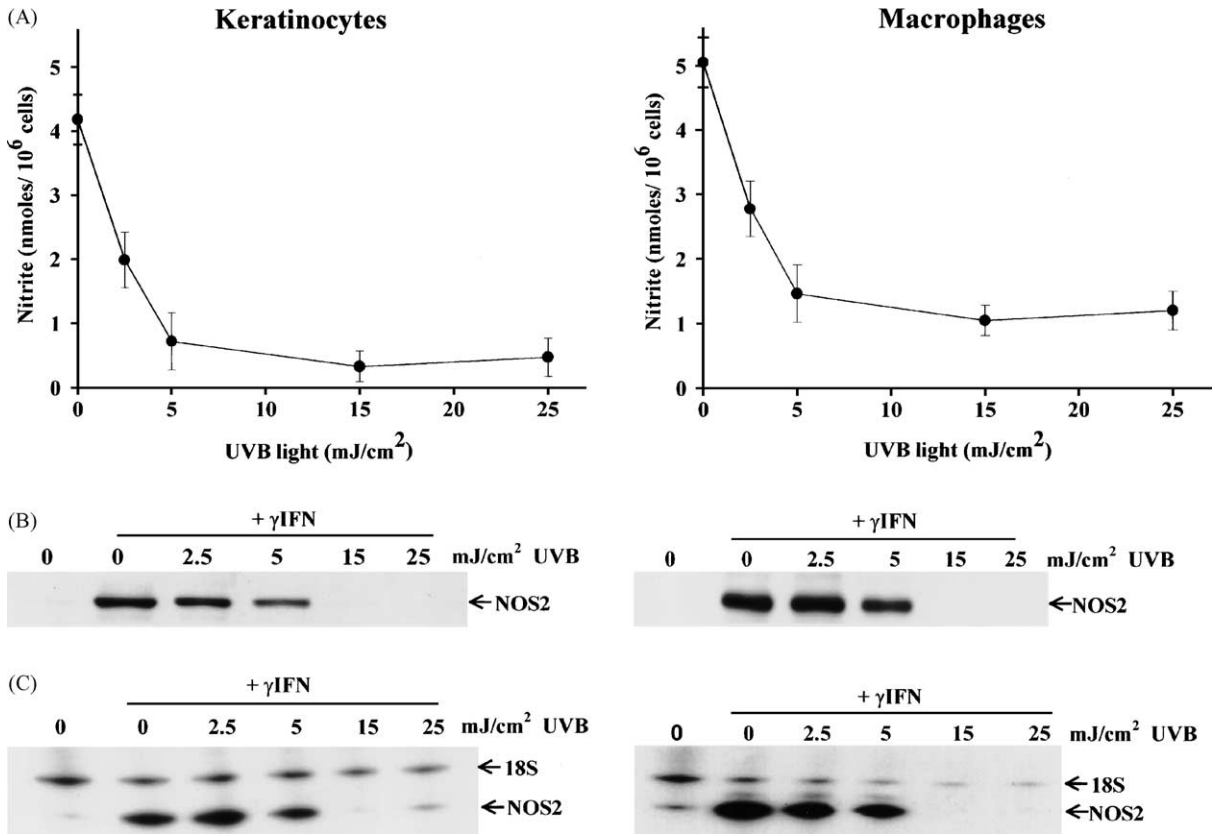

Fig. 1. Effects of UVB light on NOS2 expression and activity in murine keratinocytes and macrophages. Cells were exposed to UVB light and then treated with IFN-γ (100 U/mL) as described in Section 2. After 24 hr, supernatants were analyzed for nitrite content, and cells for NOS2 mRNA and protein expression. (A) Effect of UVB light on nitrite production induced by IFN-γ in keratinocytes and macrophages; values represents means ± SEM (N = 3). (B) Western blots showing decreases in IFN-γ-induced NOS2 protein expression in cells treated with UVB light. (C) RT-PCR showing decreased IFN-γ-induced NOS2 mRNA expression in cells treated with UVB light. 18S RNA served as an internal control. Lane U refers to control cells that had not been treated with IFN-γ.

Antioxidants such as GSH are important in protecting cells from reactive oxygen intermediates [26]. To determine if GSH was protecting against the suppression of NOS2 by UVB light, cells were depleted of this thiol tripeptide using BSO, a γ-glutamylcysteine synthetase inhibitor [27]. Exposure of BSO-treated keratinocytes and macrophages to UVB light caused a 2-fold increase in the formation of hydroperoxides when compared with cells not treated with the inhibitor (not shown). Moreover, GSH-depleted cells were found to be more sensitive to the inhibitory effects of UVB light on IFN-γ-induced NOS2 expression (Fig. 3B). These data suggest that reactive oxygen intermediates contribute to the inhibitory actions of UVB light on NOS2 and that GSH may protect against this effect.

3.2. Effects of UVB light on MAP kinase signaling

Previous studies have demonstrated that MAP kinases are important in regulating cytokine-induced NOS2 activity [28]. We found that keratinocytes and macrophages constitutively expressed the p44/p42 MAP kinase (ERK1/2), the c-JUN-N-terminal kinase (JNK), and the p38 MAP kinase (Fig. 4). Although IFN-γ by itself had no effect on the expression of these kinases, UVB light stimulated phosphorylation of JNK and p38 kinases at doses that inhibited nitric oxide production (5–25 mJ/cm^2) (Fig. 4 and not shown). Keratinocyte JNK was found to be more sensitive to UVB light than the macrophage enzyme. In contrast, ERK1/2 was constitutively phosphorylated in both cell types, and no significant changes in its activity were observed following UVB light treatment (Fig. 4). The effects of UVB light on the MAP kinases were similar in both the absence and presence of IFN-γ (not shown).

3.3. Modulation of NF-κB and Stat1 signaling

Two key transcription factors known to be important in modulating expression of the *NOS2* gene are NF-κB and Stat1 [23,24]. We found that NF-κB was constitutively activated in macrophages and keratinocytes (Fig. 5A). These data are consistent with the finding that the p50 and p65 subunits of NF-κB were localized in the nucleus of both cell types (Fig. 5B and not shown). Treatment with LPS, which was used as a positive control, but not treatment with IFN-γ, was found to enhance NF-κB nuclear binding activity in the cells (Fig. 5A and not shown). However, neither LPS nor IFN-γ caused alterations in nuclear localization of the NF-κB subunit proteins

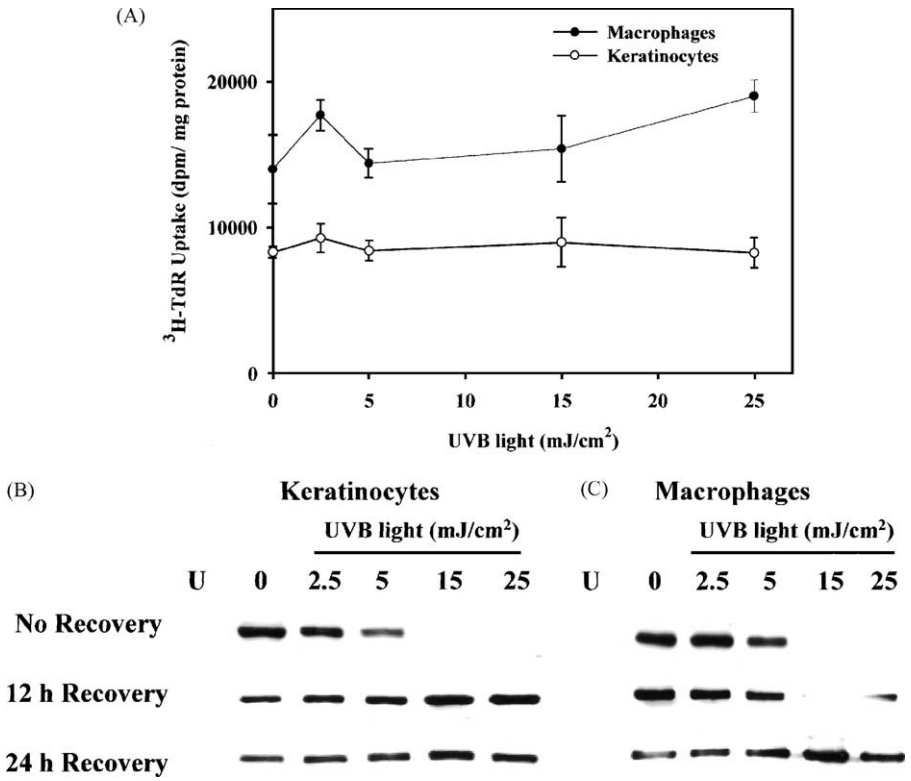

Fig. 2. [^{3}H]TdR uptake and expression of NOS2 protein in keratinocytes and macrophages following UVB light treatment. (A) Effects of UVB light on DNA synthesis. Cells were treated with UVB light and then pulse-labeled in culture medium containing [^{3}H]TdR and IFN-γ. Values shown are the average dpm/mg protein ± SEM (N = 3). (B and C) Western blots showing the recovery of NOS2 protein in keratinocytes and macrophages, respectively. Cells were treated with IFN-γ (100 U/mL, 24 hr) immediately and 12 or 24 hr after exposure to UVB light. Lane U refers to control cells that were not treated with IFN-γ.

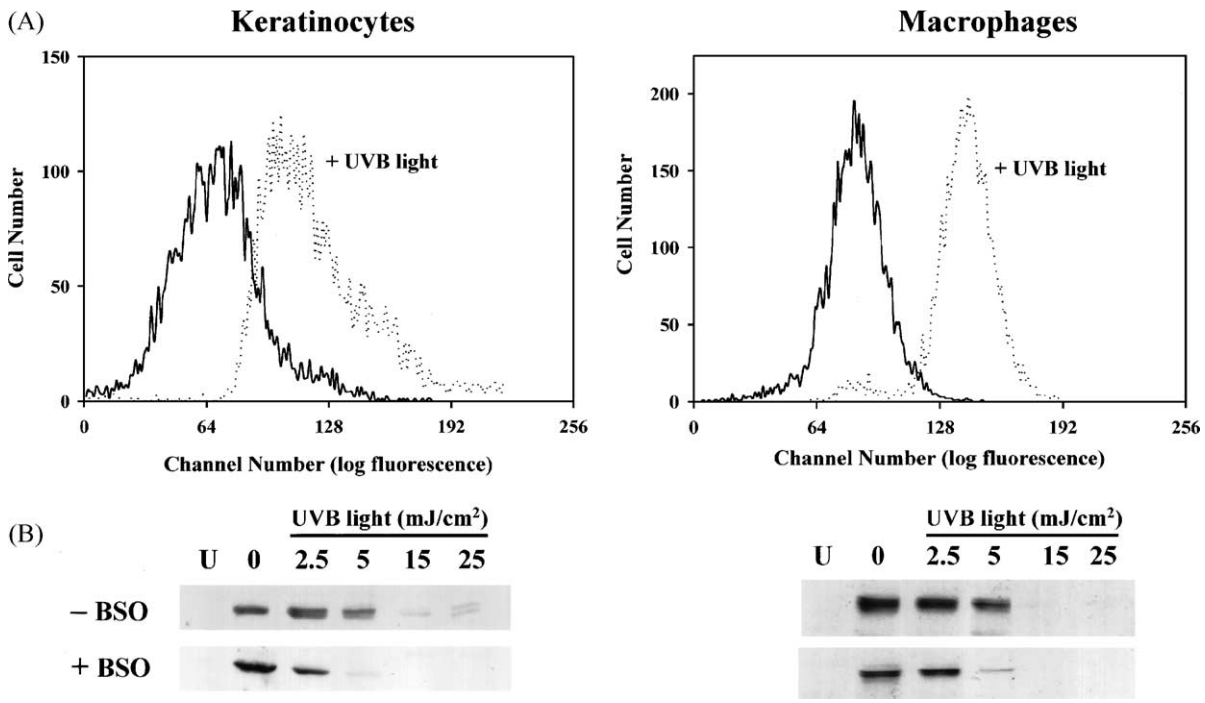

Fig. 3. Effects of UVB light on hydroperoxide production and NOS2 expression. (A) Formation of hydroperoxides in keratinocytes and macrophages exposed to UVB light. Cells were incubated with DCFH-DA (15 min, 1 μM), treated with UVB light (25 mJ/cm^2), and then analyzed for fluorescence intensity by flow cytometry. Data are presented as channel number on a four decade log scale. (B) Effects of GSH depletion on NOS2 expression. Cells were preincubated with and without BSO (100 μM, 6 hr), exposed to increasing doses of UVB light, and then treated with IFN-γ (100 U/mL). After 24 hr, cell lysates were prepared and analyzed for NOS2 content by western blotting. Lane U, control cells that were not treated with IFN-γ. For each panel, experiments were repeated three times with similar results. One representative experiment is shown.

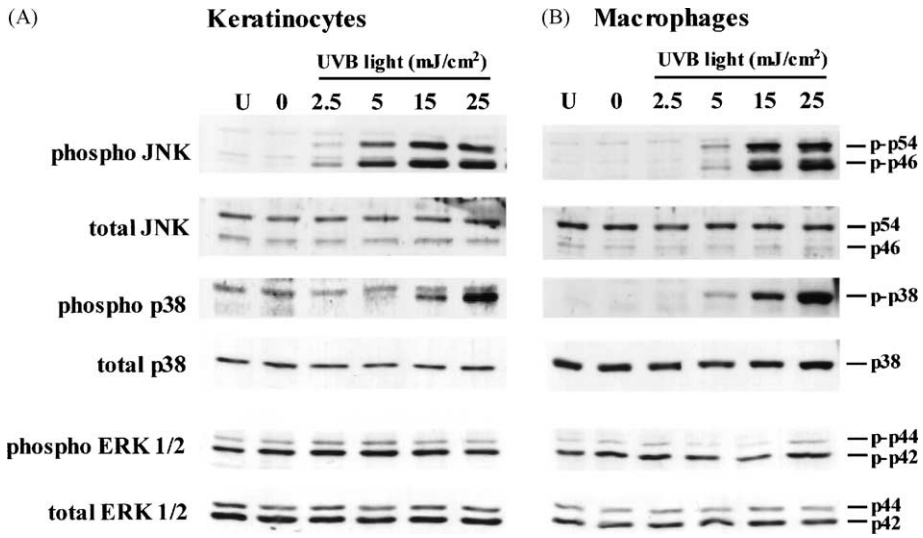

Fig. 4. Effect of UVB light on MAP kinase expression. Keratinocytes (A) and macrophages (B) were treated without and with UVB light followed by IFN-γ (100 U/mL, 30 min) and then analyzed for the total and phosphorylated forms of JNK, p38 kinase, and ERK1/2 kinase expression by western blotting. Lane U, control cells that were not treated with IFN-γ. For each panel, experiments were repeated three times with similar results. One representative experiment is shown.

(Fig. 5B and not shown). In both keratinocytes and macrophages, a supershift of the two NF-κB complexes was evident following treatment of nuclear extracts with antibodies to the p50 subunit of NF-κB. Antibodies to the p65 subunit caused a reproducible reduction in the appearance of the NF-κB complex of slowest mobility. These data are consistent with other reports indicating that the NF-κB complex of faster mobility consisted of homodimers of p50, while the slow migrating complex contained p50/p65. An unlabeled probe also prevented binding to the labeled probe, indicating that the binding was specific. In keratinocytes, UVB light caused a 20–25% reduction in constitutive NF-κB binding activity at the highest doses (15–25 mJ/cm^2) (Fig. 5A). In contrast, in macrophages, UVB light blocked NF-κB activity at all doses that were effective in inhibiting expression of NOS2 protein (Fig. 5A). UVB light had no effect on nuclear localization of the p50 and p65 subunits of NF-κB except at the highest dose (25 mJ/cm^2). Under these conditions, most of the NF-κB subunits were retained in the cytoplasm (Fig. 5B).

In both keratinocytes and macrophages, Stat1 protein was constitutively expressed. IFN-γ readily induced tyrosine phosphorylation of Stat1 and Stat1 nuclear DNA binding activity in both cell types (Fig. 6). In antibody assays, anti-Stat1 caused a supershift in the GAS–Stat1 complexes formed in both keratinocytes and macrophages, although the supershifted complex from keratinocytes was consistently less visible than that observed from macrophages. An unlabeled probe also inhibited binding to the labeled probe, indicating that the binding was specific. Whereas UVB light had no major effects on the expression of total or phospho-Stat1 protein, it caused a 20–25% reduction in Stat1 nuclear binding activity in both cell types (Fig. 6B). Small variations in the expression of phospho-Stat1 in the cells may be due to UVB light-induced alterations in phosphatase activity.

Previous studies have demonstrated that the transcription factor AP-1 acts in concert with NF-κB to regulate expression of the *NOS2* gene [29]. We found that AP-1, like NF-κB, was constitutively expressed in both keratinocytes and macrophages. Neither IFN-γ nor LPS altered this activity (Fig. 7 and not shown). Antibodies to c-JUN completely blocked AP-1 DNA binding in macrophages and partially reduced this activity in keratinocytes. An unlabeled probe also prevented binding to the labeled probe, demonstrating that the binding was specific. UVB light had no major effects on AP-1 binding activity in either cell type (Fig. 7A and B).

3.4. Effect of UVB light on expression of NOS2 reporter constructs

We next examined NOS2 expression in transient transfection assays using NOS2 promoter/enhancer wild-type and mutant luciferase reporter constructs. The constructs used included deletions in the enhancer/basal promoter and mutations in the GAS and ISRE regulatory elements and in two κB elements (κBI and κBII) (see Fig. 8A for a summary of the constructs). For these studies, transfection of the reporter constructs into keratinocytes was compared to similar studies performed previously with RAW 264.7 macrophages [23,24]. We found that luciferase activity could readily be detected in the cells following transfection of the wild-type NOS2 reporter (Fig. 8B). In both keratinocytes and macrophages, GAS and ISRE mutations (GAS$_m$ and ISRE$_m$) reduced luciferase activity by approximately 50%, suggesting that these two regulatory elements were essential for optimal *NOS2* gene

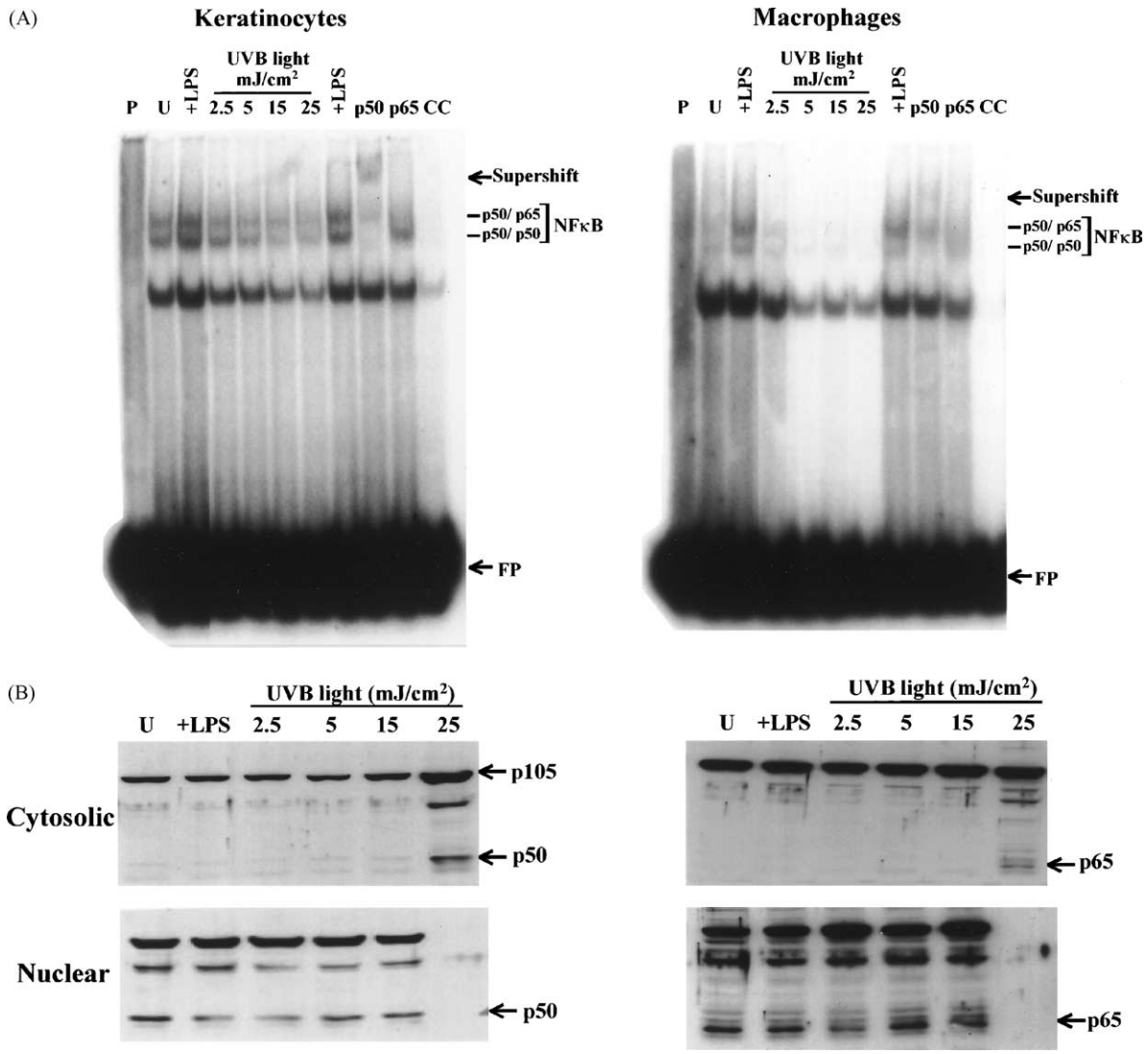

Fig. 5. Effects of UVB light on NF-κB activity. (A) NF-κB nuclear DNA binding activity in keratinocytes and macrophages. Nuclear extracts were prepared from cells 30 min after treatment with UVB light and analyzed for NF-κB binding activity in electrophoretic mobility shift assays. Extracts from cells treated with LPS (1 μg/mL, 30 min) were used as a positive control. Lane P, labeled probe alone. Lane U, untreated cells. The p50 and p65 lanes show supershifts and blocking of nuclear extracts from LPS-treated cells using antibodies to these proteins. CC, nuclear extracts run in the presence of a 100-fold excess of unlabeled probe. FP, unbound free probe. (B) Western blots showing nuclear and cytosolic expression of p50 and p65 subunits of NF-κB in keratinocytes. The p50 antibody also reacted with p105, a precursor of p50 subunit. For each panel, experiments were repeated three times with similar results. One representative experiment is shown.

expression. Similarly, both κB elements were needed for optimal activity (Fig. 8B, κBI_m and κBII_m). However, the κBI element present in the basal promoter region of the *NOS2* gene appeared to be more important in keratinocytes than macrophages for optimal gene expression. These findings are consistent with our observation that the H/Bx construct, in which the κBI site-containing basal promoter was deleted, showed greater inhibition of luciferase activity in keratinocytes than in macrophages (Fig. 8B). In contrast, the κBII region appeared to be more important in the macrophages than in the keratinocytes. The activity of the κBII_m construct paralleled that of the construct containing a deletion of all DNA upstream of the basal promoter (Bx/H) (Fig. 8B). The P/H construct, in which all of the upstream regulatory elements were deleted leaving only the TATA box and the transcriptional start site, served as a negative control in both of the cell lines. Of interest was our finding that, under identical conditions, UVB light treatment reduced NOS2 wild-type promoter reporter activity in the macrophages, while it increased promoter reporter activity in the keratinocytes (Fig. 8C and D). These data indicate that UVB light regulates *NOS2* gene promoter activity in the two cell types by distinct mechanisms.

4. Discussion

Previous studies have demonstrated that UVB light can up- or down-regulate a variety of gene products, some of which are known to be altered in skin inflammation and/or

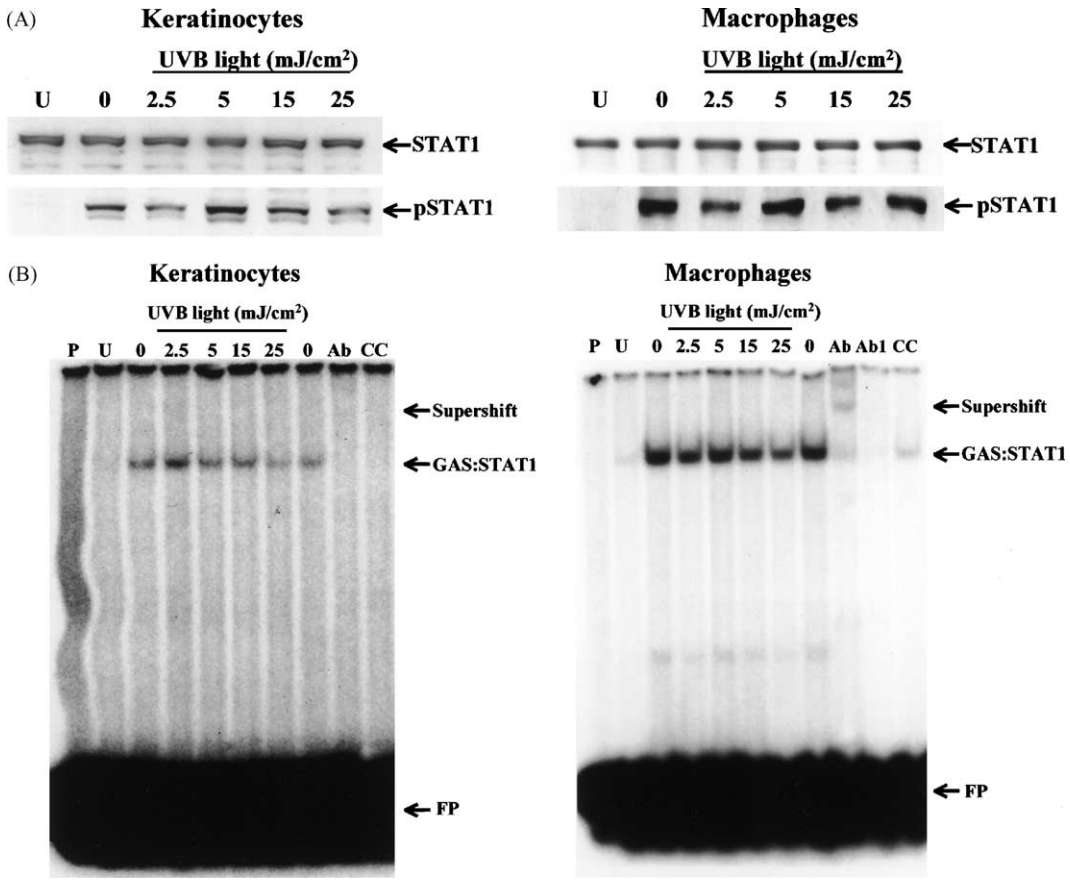

Fig. 6. Effects of UVB light on Stat1. Cells were treated with UVB light followed by IFN-γ (100 U/mL, 30 min). Cell lysates or nuclear extracts were prepared and analyzed for Stat1 protein expression or Stat1 DNA binding activity, respectively. (A) Western blots showing effects of UVB light on total Stat1 and phospho-Stat1 expression in keratinocytes and macrophages. Lane U, cells not treated with IFN-γ or UVB light. (B) Electrophoretic mobility shift assays showing Stat1 DNA binding activity in keratinocytes and macrophages. Lane P, labeled probe alone. In lanes Ab and Ab1, pStat1 antibody or total Stat1 antibody, respectively, was added to the nuclear extracts for supershift assays. CC, nuclear extracts run in the presence of a 100-fold excess of unlabeled probe. FP, unbound free probe. For each panel, experiments were repeated three times with similar results. One representative experiment is shown.

epidermal proliferative disorders like psoriasis and atopic dermatitis. For example, UVB light has been reported to up-regulate expression of COX-2 [30], ornithine decarboxylase [31], p53 [32], urokinase type plasminogen activator receptor [33], thioredoxin [34], TGF-β1 [35], c-*jun* and c-*fos* [36], TNF-α [37], IL-1 [38], IL-6 [39], IL-8 [40], and IL-10 [41], but to down-regulate the chemokine receptor CXCR-2 [42], topoisomerase IIβ-binding protein, differentiation-dependent A4 protein, the mRNA nuclear export gene product XPO1 [43], the keratinocyte growth factor receptor (KGFR) [44], and the anti-apoptotic protein Bcl-2 [45]. In the present studies, we demonstrated that UVB light also suppresses expression of NOS2 induced by the proinflammatory cytokine IFN-γ. Thus, in the range of 2.5–25 mJ/cm^2, UVB light caused a dose-dependent inhibition of IFN-γ-induced expression of NOS2 mRNA and protein, and nitric oxide production in keratinocytes and macrophages. These findings were novel since most inhibitors of nitric oxide function by interfering with nitric oxide synthase enzyme activity [13]. Inhibition of NOS2 expression was not due to cytotoxicity and was readily reversible, with keratinocytes recovering more rapidly than macrophages. The mechanisms mediating this recovery are not known and presumably reflect repair of signaling pathways modified by UVB light that regulate IFN-γ-induced expression of NOS2. Differences in recovery time between keratinocytes and macrophages may be due to the different sites of action of UVB light in the two cell types (see further below).

It is generally accepted that UVB light irradiation generates a significant oxidative challenge to the skin [46]. Indeed, numerous studies have provided evidence for the formation of reactive oxygen intermediates in skin cells following exposure to ultraviolet light [46,47]. This is consistent with our findings that UVB light readily induced the formation of hydroperoxides in keratinocytes and macrophages. The fact that reducing cellular levels of GSH, an intracellular antioxidant, caused a marked enhancement of the inhibitory effects of UVB light on NOS2 expression provides support for the concept that reactive oxygen intermediates are important in the mechanism of action of UVB light. Reactive oxygen intermediates have limited stability in cells and are highly reactive. Free radical reactions generated by these oxidants

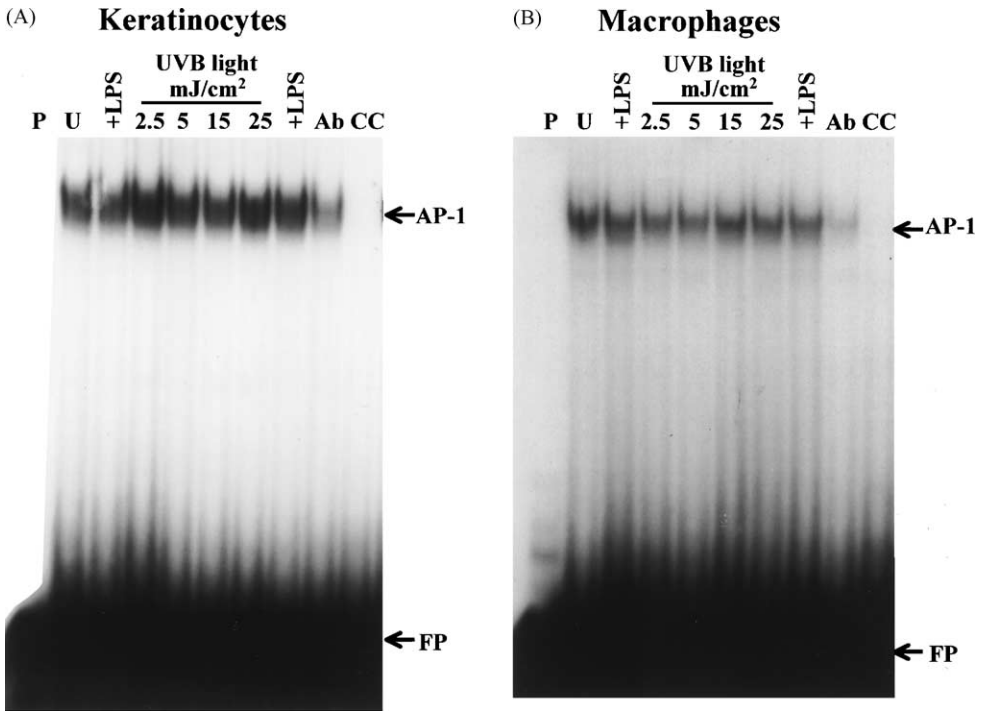

Fig. 7. Effects of UVB light on AP-1. Electrophoretic mobility shift assays of AP-1 DNA binding activity in nuclear extracts of keratinocytes (A) and macrophages (B). Cells treated with LPS (1 μg/mL, 30 min) were used as a control. Lane P, labeled probe alone. Lane U, cells not treated with IFN-γ or UVB light. In lane Ab, antibodies against c-Jun, a component of the AP-1 complex, were added to nuclear extracts for supershift assays. The Ab and the CC lanes were treated with LPS. CC, nuclear extracts run in the presence of a 100-fold excess of unlabeled probe. FP, unbound free probe. For each panel, experiments were repeated three times with similar results. One representative experiment is shown.

are potentially damaging to cells and tissue [48]. However, reactive oxygen intermediates also participate in many normal physiologic events including antibacterial defense, signal transduction, cell growth regulation, differentiation, and apoptosis [49]. These latter activities may be important in epidermal diseases such as psoriasis and atopic dermatitis where alterations in cell growth and differentiation, as well as abnormal production of cytokines and growth factors are hallmarks of the diseases and represent potential targets for reactive oxygen intermediates generated by UVB light [49,50].

Several recent studies have examined the effects of UVB light on nitric oxide synthase in keratinocytes. In these studies, increased human keratinocyte nitric oxide synthase activity has been reported in the absence of cytokines following treatment of cells with up to 50 mJ/cm^2 UVB light [51,52]. This was thought to be due to increased activity of the low output brain isoform of nitric oxide synthase (NOS1) [53,54]. In normal human skin and the skin of systemic lupus erythematosus patients, UVB light has also been reported to stimulate NOS2 expression after prolonged periods of time (1–3 days), while in murine skin, UVB light increases NOS2 in infiltrating leukocytes and nitric oxide in both the dermis and epidermis after 2 days [51]. In contrast, UVB light has been reported to suppress cytokine-induced NOS2 expression in a murine keratinocyte cell line, although the mechanisms were not investigated [55]. In our studies, UVB light alone had no effect on nitric oxide production in either keratinocytes or macrophages, suggesting that it does not activate other isoforms of nitric oxide synthase.

A question arises as to the mechanism by which UVB light suppresses NOS2 in keratinocytes and macrophages. Presumably, UVB light interferes with signaling processes that lead to expression of the NOS2 message. To test this possibility, we initially analyzed the MAP kinases since these proteins are known to be important in modulating the expression of many UVB light-sensitive genes including *NOS2* [56]. Moreover, in a variety of cell types, irradiation with UVB light activates the p44/p42 MAP kinase (ERK1/2), the c-JUN-N-terminal kinase (JNK), and the p38 MAP kinase [57–60]. Although IFN-γ had no effect on the MAP kinases in keratinocytes and macrophages, JNK and p38 kinases, but not p44/p42 kinase, were activated by UVB light. These data suggest that the JNK and p38 kinases provided negative regulatory signals for the expression of NOS2, potentially by direct interference with cytokine signaling or by activating an inhibitor of NOS2 expression. This is supported by the finding that the peroxisome proliferator-activated receptor-γ (PPAR-γ), a substrate for the MAP kinases that can regulate its activity [61–63], readily inhibits induction of NOS2 in macrophages [64–66]. This is due, at least in part, to blocking of the activities of the transcription factors AP-1, Stat1, and NF-κB [65,66]. Similarly, the JNK cascade regulates expression of Smad7, an important mediator of the transcriptional

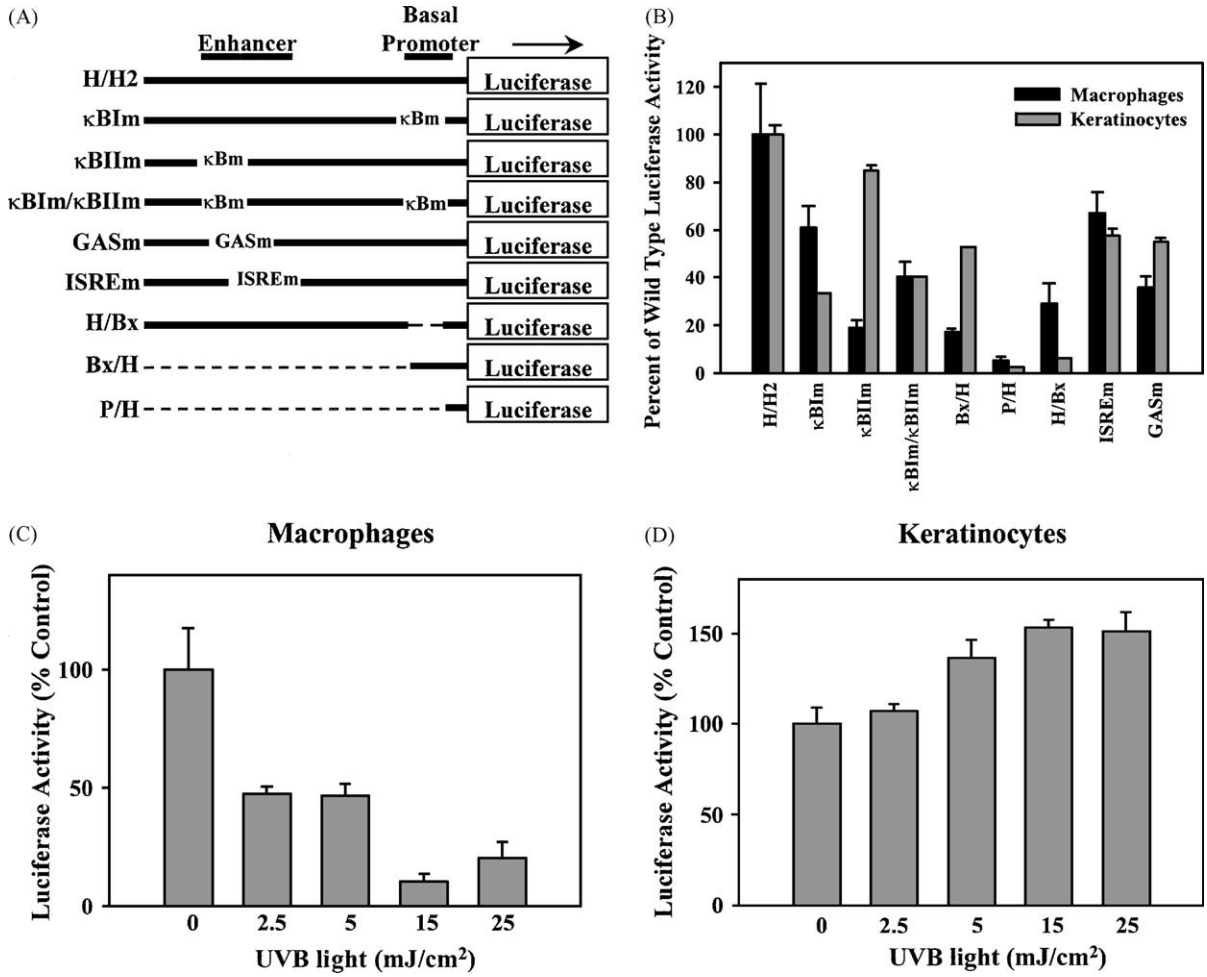

Fig. 8. Transfection of wild-type and mutant NOS2 luciferase promoter constructs into macrophages and keratinocytes. (A) Schematic representation of the various promoter mutant luciferase constructs (adapted from Ref. [23,24]). H/H2 represents the wild-type NOS2 promoter construct, κBI_m represents the mutation of the basal NF-κB binding site, κBII_m represents the mutation of the enhancer linked NF-κB binding site, $\kappa BI_m/\kappa BII_m$ represents the mutated construct for both NF-κB binding sites, GAS_m represents the mutated construct for the GAS element, $ISRE_m$ represents the mutated construct for the ISRE element, H/Bx represents the deletion of the entire basal promoter, Bx/H represents the deletion of the entire enhancer promoter, and P/H represents the deletion of all the upstream regulatory elements leaving only the transcriptional start site and the TATA box. (B) Comparison of luciferase activity in keratinocytes and macrophages following transfection with promoter plasmids. Data are shown as a percentage of wild-type promoter activity of the ratio of the firefly luciferase activity/*Renilla* luciferase activity. Values represent means ± SEM (N = 3). Data from the macrophages were adapted from Ref. [23,24]; macrophages, black bars; keratinocytes, grey bars. (C and D) Effects of UVB light on luciferase activity from wild-type reporter constructs in macrophages and keratinocytes, respectively. Cells were transiently transfected with the wild-type NOS2 reporter plasmid along with a *Renilla* luciferase control, treated with UVB light, and then stimulated with IFN-γ (100 U/mL) and LPS (1 μg/mL). After 6 hr, cell lysates were prepared and analyzed for luciferase activity. Values represent the means ± SEM (N = 3) from one set of three similar experiments.

effects of TGF-β [67] that is up-regulated by UVB light [68]. The related Smad3 has been shown recently to be an effective inhibitor of the induction of NOS2 in macrophages [69]. The JNK and p38 MAP kinases also directly modulate transcription factors that are important in both positive and negative control of *NOS2* gene expression [28,56].

Sequence analysis of the promoter for the *NOS2* gene in RAW 264.7 macrophages has revealed the presence of a number of consensus motifs for transcription factors such as NF-κB, Stat1, and AP-1 [70–72]. Optimal expression of the *NOS2* gene is known to require binding of both NF-κB and Stat1 [70]. Furthermore, as indicated above, AP-1 can act in concert with NF-κB to regulate *NOS2* expression [29]. Constitutive NF-κB binding activity was detected in keratinocytes and macrophages. This activity was inhibited by UVB light. Macrophages were significantly more sensitive to the inhibitory effects of UVB light than were keratinocytes. In contrast, UVB light had no effect on nuclear localization of the p50 and p65 subunits except at the highest dose (25 mJ/cm^2). Previous studies have shown that UVB light activates NF-κB in human keratinocytes and fibroblasts [73,74], possibly via activation of the tumor necrosis factor receptor-1 [74]. Constitutive AP-1 DNA binding activity was also observed in both keratinocytes and macrophages, and this activity was not altered by UVB light treatment. An increase in AP-1 DNA binding activity has been described previously by UVB light in JB6 murine

keratinocytes [75]. Differences between our results on AP-1 and NF-κB binding activity and those reported by others may be due to the distinct origin of the cells and/or the presence of inducible vs. constitutive transcription factor activity. The mechanisms regulating constitutive DNA binding activity of NF-κB and AP-1 in keratinocytes and macrophages used in the present study are not known. As indicated above, many cytokines are localized in skin tissue during inflammatory dermatosis, and some of these can induce NF-κB and AP-1. Continuous exposure of cells to these cytokines may cause constitutive expression of transcription factors *in vivo*. In this regard, in *IκBα* knock-out mice, constitutive expression of NF-κB is observed and these animals display a widespread skin inflammation resembling atopic dermatitis [76]. Thus, our studies with cells constitutively expressing NF-κB may be particularly relevant in elucidating the responses of cells from psoriasis and atopic dermatitis to UVB light.

In contrast to NF-κB and AP-1, Stat1 phosphorylation and subsequent DNA binding to GAS were induced by IFN-γ in both keratinocytes and macrophages. Although no effects were observed on Stat1 phosphorylation, UVB light partially (20–25%) inhibited Stat1 DNA binding activity. These data are consistent with an earlier report demonstrating inhibition of IFN-γ-induced Stat1 binding to GAS in mouse keratinocytes treated with 25 mJ/cm^2 UVB light [77]. These investigators also found that UVB light had no effect on either JAK-1 or JAK-2 kinase activity, suggesting that this treatment did not down-regulate the IFN-γ receptor [77].

Based on the above findings, it appears that inhibition of NF-κB and Stat1 may contribute to the inhibitory effects of UVB light on expression of the *NOS2* gene. To test this possibility, we analyzed expression of NOS2 promoter/luciferase reporter constructs in the cells [23,24]. As expected, keratinocytes, like macrophages, were found to require κB elements in both the basal and enhancer regions of the promoter for maximal activity. GAS and ISRE elements were also needed for optimal *NOS2* gene expression in the cells. Several differences were noted between the two cell types including the fact that the κB element in the basal promoter region appeared to be more important for optimal gene expression in keratinocytes than in macrophages, while the reverse was true for the κB element in the enhancer region. Surprisingly, with the wild-type promoter, the two cell types responded differently to UVB light treatment. Whereas in macrophages, UVB light inhibited expression of the reporter construct, an enhancement of luciferase reporter activity was noted in the keratinocytes. These data indicate that UVB light regulates *NOS2* gene promoter activity in the two cell types by distinct mechanisms. In macrophages, reduced Stat1 and NF-κB DNA binding to the *NOS2* promoter following UVB light treatment is likely to be important for inhibition of its activity, and this may limit *NOS2* expression. In contrast, since *NOS2* expression is blocked in keratinocytes, the partial inhibitory effects of UVB light on NF-κB and Stat1 binding are not sufficient to reduce promoter activity. The inhibitory effects of UVB light on *NOS2* gene expression must occur downstream of the *NOS2* promoter or at regulatory elements located outside of the *NOS2* promoter/enhancer regions that have yet to be characterized.

In summary, the present studies demonstrated that UVB light inhibits IFN-γ-induced expression of NOS2 mRNA and protein, and nitric oxide production in two cell types known to be important in skin inflammation. In both cell types, UVB light could interfere with NF-κB and Stat1 DNA binding activity. However, the effects were more pronounced in macrophages, which was consistent with the inhibitory effects of UVB light on NOS2 promoter reporter activity in this cell type. Unexpectedly, NOS2 promoter reporter activity was enhanced by UVB light in keratinocytes. These data suggest that UVB light functions to inhibit *NOS2* gene expression by different mechanisms in the two cell types. Further studies characterizing the regulation of expression of the *NOS2* gene in these cells are needed to identify potential targets of UVB light.

Acknowledgments

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