UV-A-induced decrease in nuclear factor- κ B activity in human keratinocytes

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Previous reports have demonstrated an increase in nuclear factor- κ B (NF- κ B) activity in response to UV radiation. These studies have essentially focused on the DNA-damaging fraction of solar UV radiation (UV-B and UV-C). In contrast, the effects of UV-A radiation (320–400 nm) on NF- κ B are not well known. In this study, we present evidence that UV-A radiation induces a marked decrease in NF- κ B DNA-binding activity in NCTC 2544 human keratinocytes. In addition, NCTC 2544 keratinocytes pretreated with UV-A fail to respond to NF- κ B inducers. Moreover, UV-A radiation induces a decrease in NF- κ B-driven luciferase reporter gene expression in NCTC 2544 keratinocytes. The expression of the gene encoding I κ B α (I κ B is the NF- κ B inhibitor), which is

closely associated with NF- κ B activity, is also reduced (3-fold) upon UV-A treatment. Our results indicate that the UV-A-induced decrease in NF- κ B DNA-binding activity is associated with a decrease in the levels of the p50 and p65 protein subunits. This is the first evidence that an oxidative stress, such as UV-A radiation, may induce a specific decrease in NF- κ B activity in mammalian cells, probably through degradation of NF- κ B protein subunits. These findings suggest that UV-A could modulate the NF- κ B-dependent gene expression.

Key words: oxidative stress, skin, transcription factors, UV radiation.

INTRODUCTION

Changes in the cellular environment frequently affect the activities of many transcription factors. Nuclear factor- κ B (NF- κ B) is an example of a transcription factor whose activity is triggered by many agents that generate reactive oxygen species, including H₂O₂ and radiation, as well as by phorbol esters (e.g. PMA) and tumour necrosis factor α (TNF α) [1–4]. NF- κ B proteins are potent inducible gene-regulatory factors that are expressed in a wide array of tissues. They are composed of homo- or heterodimers of Rel-family proteins. In most cell types, NF- κ B resides in the cytoplasm in a latent form composed of subunits of 50 kDa and 65 kDa that are associated with the specific inhibitor of NF- κ B (I κ B). In responses to multiple stress inducers, the inhibitor is inactivated and NF- κ B proteins are translocated to the nucleus, where they activate their target genes [5–7].

The skin comes in contact with a variety of agents that can induce oxidative stress, and is now increasingly exposed to sunlight. UV radiation can trigger different signals and effects in skin cells, causing photo-oxidative damage resulting in numerous pathologies, including dermatitis, skin cancer and skin photo-aging [8,9]. Many reports have focused on the effects of UV-B (290–320 nm) and UV-C (< 290 nm) radiation on factors that regulate gene expression, since such radiation is known to damage DNA directly [10,11]. In contrast, the action of UV-A (320–400 nm) on these factors is poorly understood. UV-A radiation represents 96 % of the total solar UV radiation reaching the earth's surface, and therefore plays an important role in dermal and epidermal photodamage, as indicated by many studies [12–14]. The effects of UV-A on cell components are thought to result from a photodynamic action producing reactive oxygen intermediates that can indirectly affect different cellular targets [15,16]. Indeed, exposure of cells to UV-A has been shown to alter the activity of various proteins in the plasma

membrane, the cytoplasm and the nucleus of the cell [17–20]. In an effort to examine whether UV-A is able to modulate the activity of transcription factors, we analysed the effects of UV-A on NF- κ B, which is known to be a prototypical oxidative-stress-responsive transcription factor. This factor plays a crucial role in the regulation of many dermal and epidermal genes that participate in the immune and inflammatory responses, as well as in cell adhesion [21,22]. These genes include those encoding cytokines (TNF α , interleukin-1), chemokines, such as interleukins-2, -6 and -8, and cell-surface receptors (e.g. intercellular cell-adhesion molecule 1).

In the present study, we first compared the DNA-binding and transcriptional activities of NF- κ B in control and UV-A-irradiated keratinocytes and fibroblasts. These experiments provided direct evidence for a marked decrease in both activities in irradiated cells. In addition, UV-A-pretreated NCTC 2544 keratinocytes were strongly impaired in their ability to activate NF- κ B DNA binding in response to NF- κ B inducers such as TNF α , PMA and UV-B. Our results indicate that this decrease in NF- κ B activity is associated with a decrease in p65 and p50 expression which could be ascribed, at least in part, to degradation of these proteins.

MATERIALS AND METHODS

Reagents

Double-stranded oligonucleotides containing the NF- κ B sequence were synthesized on the 0.2 μ mol scale by Eurogentec. [γ -³²P]ATP was purchased from ICN. T₄ polynucleotide kinase was obtained from Boehringer Mannheim. The Oct-1 consensus oligonucleotide was synthesized by Promega. Recombinant human TNF α was purchased from Cliniscience. All other reagents were obtained from Sigma.

Abbreviations used: NF- κ B, nuclear factor- κ B; I κ B, inhibitor of NF- κ B; AP-1, activator protein-1; TNF α , tumour necrosis factor α ; TBARS, thiobarbituric-acid-reactive substances; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay.

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Cell culture

The NCTC 2544 human keratinocyte cell line was purchased from ICN. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. At 24 h prior to UV-A irradiation, cells were growth-arrested at subconfluence by incubation with serum-free DMEM supplemented with 0.1% (w/v) BSA.

EJ2 rat fibroblasts (kindly provided by Dr. J. de Gunzburg, Institut Curie, Paris, France) and W4 rat fibroblasts (generously provided by Dr. S. Yamaoka, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Japan) were cultured in DMEM (4.5 g/l glucose) supplemented with 10% (v/v) FCS, 2 mM glutamine and antibiotics as above.

UV irradiation

UV-A irradiation was carried out using a Vilber Lourmat table equipped with a TF-20L tube, emitting maximally at 365 nm, at a dose rate of 4.0 ± 0.2 mW/cm². Our irradiation equipment has been designed to avoid UV-B contamination. A piece of glass (4 mm thickness) was placed 20 mm above the table to absorb UV-B light (transmittance < 0.01% at 310 nm). Before irradiation, cell monolayers were washed three times with PBS and covered with Hank's salt solution. After exposure to UV-A doses up to 240 kJ/m², the irradiation medium was replaced by serum-free medium until preparation of nuclear extracts. Sham-irradiated control cells were treated in a similar way, but were not irradiated. Sodium azide (50 mM in Hank's salt solution) was present only during irradiation of cells. For irradiation in the presence of ²H₂O, deuterium oxide (99.9 atom % ²H) was used at a final concentration of 95% in Hank's salt solution.

The source of UV-B (312 nm) was a TFX-35 illuminating table (Vilbert-Lourmat) equipped with six 15 W UV-B lamps, with an emission spectrum in the range 290–320 nm. UV-B was delivered after removal of the glass windows and the lids of the dishes. The fluence of the UV-B table, at the level of the cells, was 12.5 W/m².

Thiobarbituric-acid-reactive substances (TBARS) measurement and effects of antioxidants

Lipid peroxidation end products (TBARS), including malondialdehyde, were measured after cell irradiation. Measurements were performed on aliquots of the medium from irradiated or sham-irradiated cells, as it has been shown previously that most of the TBARS produced after UV-A exposure are secreted by cells [23]. The samples were assayed for TBARS using the fluorimetric method of Yagi [24], with minor technical modifications. In some experiments, the effect of the antioxidant vitamin E was tested. This antioxidant was introduced at 50 µM to cultures 24 h prior to irradiation.

Preparation of nuclear extracts

Nuclear extracts were prepared as described by Dignam et al. [25], with minor modifications. Briefly, NCTC 2544 keratinocytes were harvested, pelleted and resuspended in 100 µl of buffer A (10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol and 0.1% Nonidet P-40) supplemented with the following protease inhibitors: 0.2 mM PMSF, 2 µM aprotinin, and 1 µg/ml each of antipain, pepstatin, benzamidin and leupeptin. After homogenization in a tight-fitting Dounce homogenizer, cell lysates were left on ice for 10 min and then centrifuged at 2000 g for 10 min. The nuclear pellet was then washed with buffer A

without Nonidet P-40, and resuspended in 50 µl of 20 mM Hepes, pH 7.9, 490 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol and 25% glycerol, and incubated in the mixture of protease inhibitors described above. The suspension was gently shaken for 30 min at 4 °C. After centrifugation at 13000 g for 15 min, the nuclear extracts (supernatants) were collected and quickly frozen in 15 µl aliquots at –80 °C.

Electrophoretic mobility shift assay (EMSA)

Either strand of the NF-κB duplex was ³²P-end-labelled with T₄ polynucleotide kinase and [γ-³²P]ATP, according to the manufacturer's protocol, and then hybridized with its complementary strand. The binding reaction was performed for 25 min at room temperature with 3–5 µg of nuclear proteins in 20 µl of 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40 and 3 µg of poly(dI-dC). The solution contained 0.1 pmol of the end-labelled double-stranded NF-κB probe (20000–40000 c.p.m.). Where indicated, the incubation mixture also contained a large excess of a double-stranded unlabelled oligonucleotide containing either the NF-κB (specific) or the Oct-1 (an unspecific double-stranded oligonucleotide of identical length) consensus sequence. The binding mixtures were electrophoresed on a 6% (w/v) non-denaturing polyacrylamide gel in 90 mM Tris, 90 mM borate and 0.1 mM EDTA, pH 8.3, dried and analysed with a Phosphorimager instrument SP (Molecular Dynamics). For the characterization of DNA-binding proteins, nuclear proteins were incubated for 30 min at 4 °C with specific antibodies directed against p50 or p65 before the binding reaction was initiated.

Western blotting

Proteins from the cytoplasmic or nuclear extracts were fractionated on an SDS/10%-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL; Amersham). Blots were then blocked overnight at 4 °C with 5% (w/v) non-fat dried milk, 0.05% Nonidet P-40 and 0.1% Tween in Tris/NaCl buffer (20 mM Tris base/137 mM NaCl, pH 7.6) and incubated for 3 h at room temperature with a 1:1000 dilution of polyclonal antibodies specific for the p65 (1226) or p50 (1141, 1157) subunits of NF-κB (generously provided by Dr. N. Rice, Laboratory of Molecular Virology and Carcinogenesis, Frederick, MD, U.S.A.). After three 15 min washes with Tris/NaCl buffer containing 0.1% Tween 20, the membranes were incubated for 3 h at room temperature with a 1:10000 dilution of an anti-rabbit peroxidase-conjugated secondary antibody. Detection of immunoreactive proteins was performed with the NEN ECL[®] Western blotting detection system.

Transient transfection procedure and luciferase assay

Cells were seeded at 50000 cells per well in 24-well dishes 24 h before transfection in 1 ml of DMEM supplemented with 10% (v/v) FCS. Immediately before transfection, cells were rinsed and supplemented with fresh serum-free culture medium. Cells were transfected using the Exgen vector (Euromedex) with one of the following plasmids: (Igκ)3-conaluc, a reporter plasmid which contains three copies of the immunoglobulin κ chain enhancer κB site upstream of the minimal conalbumin promoter fused to the luciferase reporter gene, or its control counterpart conaluc (generously provided by Dr. A. Israël, Institut Pasteur, Paris, France). After transfection, cells were incubated for 36 h in DMEM supplemented with 0.3% (v/v) FCS; they were then irradiated or not with a 240 kJ/m² dose of UV-A and harvested 8 h after treatment. Where indicated, cells were treated either for

8 h in the presence of $\text{TNF}\alpha$ or for 1 h with PMA, and were harvested at the same time as the UV-A-treated cells. The luciferase assay was monitored by using a commercial kit (Promega) and by photon counting (Biolumat, a commercial LB9500 instrument; Berthold, Nashua, NH, U.S.A.). Each transfection experiment was done in triplicate, and results are expressed as mean light units/mg of cell protein [measured using the BCA assay (Pierce)].

Northern blot analysis

Total RNA (15 μg) was electrophoresed in a 1.2 % (w/v) agarose gel and transferred to a Hybond-C nitrocellulose membrane (Amersham). The membranes were hybridized overnight at 42 °C with the probe labelled with [α - ^{32}P]dCTP using the Megaprime DNA labelling system (Amersham). The hybridized membranes were washed and exposed to Hyperfilm-MP (Amersham). The blot of RNA was stripped by boiling in 0.1 % (w/v) SDS and probed again with a β -actin probe to confirm equal loading of RNA samples.

RESULTS

Decreased NF- κ B DNA-binding activity in UV-A-irradiated NCTC 2544 keratinocytes

In a first set of experiments, nuclear extracts prepared from control and irradiated cells were analysed by EMSA using an oligonucleotide containing a κ B site. Figure 1(A) shows the effects of increasing UV-A doses on the formation of NF- κ B–DNA complexes in human keratinocyte extracts. The extent of the decrease in binding was dose-dependent, with a 4–5-fold decrease at the maximal UV-A dose (240 kJ/m^2). It is noteworthy that the untreated NCTC 2544 keratinocytes exhibited a signi-

Figure 1 Dose-dependence of the UV-A-induced decrease in NF- κ B fixation activity

(A) NCTC keratinocytes were stimulated with increasing UV-A doses. Nuclear extracts were prepared 3 h after UV-A treatment. Portions of 5 μg of the nuclear fractions were assayed for NF- κ B DNA-binding activity, as described in the Materials and methods section. (B) Characterization of the NF- κ B DNA-binding complexes. Nuclear extracts (15 μg) were prepared from NCTC cells irradiated with 240 kJ/m^2 UV-A and treated with anti-p50 (lane 2) or anti-p65 (lane 3) antibodies, or with a 50-fold excess of an unlabelled oligonucleotide containing NF- κ B sites (lane 4) or a 100-fold excess of non-specific competitor Oct-1 consensus sequence (lane 5). NF- κ B complexes are indicated, and supershifted complexes are denoted (a and b). (C) Nuclear fractions (5 μg) were prepared from NCTC cells irradiated with 240 kJ/m^2 UV-A (same extract as in A) and assayed for AP-1 DNA-binding activity. Identical results were observed in three independent experiments.

Figure 2 UV-A impairs the activation of NF- κ B in response to the NF- κ B inducers $\text{TNF}\alpha$, PMA and UV-B

NCTC cells were irradiated (+) or not (–) with UV-A (240 kJ/m^2) and then stimulated with $\text{TNF}\alpha$ (3 h; 10 ng/ml), PMA (1 h; 100 nM) or UV-B (500 J/m^2) (lane Ø, control). Nuclear extracts were prepared 3 h after treatment with UV-A and assayed for NF- κ B DNA-binding activity, as described in the Materials and methods section. Identical results were observed in four independent experiments.

ficant level of NF- κ B DNA binding. The specificity of the UV-A-mediated decrease in NF- κ B DNA binding was assessed in a competition experiment: the NF- κ B complex disappeared in the presence of unlabelled oligonucleotide. In contrast, this complex remained unchanged in the presence of a large excess of an unlabelled oligonucleotide containing the unrelated Oct-1 consensus sequence (Figure 1B, lanes 4 and 5). Supershift analysis using specific antibodies to the p50 and p65 subunits indicated that the complex is composed of both p65 and p50 proteins (Figure 1B).

We also analysed the nuclear extracts prepared from irradiated NCTC 2544 keratinocytes (UV-A dose of 240 kJ/m^2) for activator protein-1 (AP-1) transcription factor DNA-binding activity. Figure 1(C) shows that, in contrast with its effect on the NF- κ B transcription factor, UV-A (240 kJ/m^2) induced a 3-fold increase in AP-1 DNA-binding activity. This dose of UV-A was used in the subsequent experiments.

UV-A-irradiated NCTC 2544 keratinocytes do not respond to NF- κ B activators ($\text{TNF}\alpha$, PMA and UV-B)

In view of previous studies which showed that PMA, $\text{TNF}\alpha$ and UV-B are potent inducers of NF- κ B DNA-binding activity in many cell types, we wanted to determine the effects of these treatments on UV-A-irradiated cells. Nuclear extracts were prepared from NCTC 2544 keratinocytes that had been treated or not with UV-A, incubated for the times indicated with the various agents and then analysed by EMSA. As shown in Figure 2, these stimuli enhanced NF- κ B DNA-binding activity to different extents in NCTC 2544 keratinocytes, indicating that these cells possessed a functional NF- κ B activator system. However, when NCTC 2544 keratinocytes had been pretreated with UV-A, they could not respond to induction of NF- κ B DNA-binding activity by $\text{TNF}\alpha$, UV-B or PMA (Figure 2).

UV-A decreases NF- κ B-dependent luciferase reporter gene expression transcriptional activity and reduces the expression of I κ B α

To determine whether UV-A decreases the level of NF- κ B transcriptional activity, NCTC 2544 keratinocytes were trans-

Figure 3 UV-A-mediated decrease in NF- κ B transcription activity

(A) NCTC keratinocytes were transfected by the Exgen method with 0.4 μ g of (Ig κ)3-conaluc reporter plasmid or its control counterpart. After transfection, cells were maintained for 36 h in a medium containing 0.3% (v/v) FCS and then treated with 240 kJ/m² UV-A, with TNF α (8 h; 10 ng/ml) or with PMA (1 h; 100 nM) (C, control). Cells were harvested 8 h after treatment for luciferase assay as described in the Materials and methods section. (B) NF- κ B-mediated luciferase reporter gene activity was also measured in H12-Ha-ras-transfected rat fibroblasts (EJ2) and fibroblasts expressing Tax protein (W4), and in their parental (P) cell lines, with or without UV-A treatment, using a procedure similar to that described above. (C) NCTC cells were irradiated or not with UV-A (240 kJ/m²) and harvested 6 h after treatment for RNA preparation. Total RNA (15 μ g) was electrophoresed in a 1.2% (w/v) agarose gel and hybridized to a ³²P-labelled I κ B cDNA probe. A β -actin probe was used to confirm comparable loading of RNA samples. C, sham-irradiated cells; UV-A, irradiated cells. Results are representative of three (A) or two (B and C) experiments.

fected with the (Ig κ)3-conaluc plasmid. At 36 h after transfection, the cells were treated or not with UV-A. As positive controls, transiently transfected NCTC cells were treated with either TNF α or PMA. As shown in Figure 3(A), treatment of NCTC cells with TNF α induced NF- κ B transcriptional activity in NCTC cells by approx. 3-fold. PMA treatment also strongly enhanced NF- κ B activity (15-fold). These results confirm the stimulatory effects of TNF α and PMA on NF- κ B transcriptional activity. In contrast, when transiently transfected NCTC cells were treated with UV-A, a 2 ± 0.4 -fold decrease in normalized luciferase activity was observed, as compared with sham-irradiated cells. These findings validate the specific decrease in NF- κ B DNA-binding activity revealed by EMSA in UV-A-exposed NCTC cells.

Subsequently, we wished to confirm that UV-A decreases NF- κ B activity in cells other than NCTC 2544 keratinocytes. To facilitate this study, we used cell lines that show a high level of basal NF- κ B activity. First, [Val¹²]Ha-ras-transfected cells were used, because previous studies have shown that these cells induce an increase in NF- κ B activity as compared with the parental cell line [26,27]. Secondly, we used fibroblast cells expressing the Tax protein (W4), which has been shown to strongly activate NF- κ B

activity [28]. As shown in Figure 3(B), normalized luciferase activity measured in Ha-ras-transfected Rat-1 cells was about 15-fold greater than the activity measured in parental Rat-1 cells, in accordance with previous studies. Interestingly, UV-A exposure of [Val¹²]Ha-ras-transfected fibroblasts induced a 2-fold decrease in their (high) level of NF- κ B activity. Similarly, Figure 3(B) shows that the high NF- κ B activity of W4 cells was decreased by about 3-fold after UV-A irradiation, as compared with sham-irradiated cells. In parallel, NF- κ B activity in parental cells was also reduced by UV-A exposure (2–3-fold). Together, these results indicate that the UV-A-induced decrease in NF- κ B activity is not a cell-type-specific effect.

To test further the functional effects of the inhibition of NF- κ B transcriptional activity, we studied the expression of a well-known NF- κ B target gene, I κ B α . [5,6]. The I κ B α gene presents potential κ B sites in its promoter region, and its expression is induced by NF- κ B [29]. Northern blot analysis (Figure 3C) shows that UV-A irradiation reduced the expression of the I κ B α gene in NCTC 2544 keratinocytes by about 3-fold. This finding is in agreement with the decrease in NF- κ B-dependent luciferase gene expression shown above, and confirms that NF- κ B-dependent gene expression may be negatively regulated by UV-A exposure.

UV-A irradiation decreases the levels of immunoreactive p65 and p50 subunits

To gain some insight into the mechanism of the UV-A-mediated decrease in NF- κ B DNA-binding activity, we analysed cytosolic extracts prepared from irradiated and control NCTC 2544 keratinocytes for NF- κ B binding activity after incubation with or without sodium deoxycholate. This procedure is known to activate latent cytoplasmic NF- κ B, as it promotes the dissociation of I κ B from the NF- κ B–I κ B complex. As shown in Figure 4(A), treatment of the cytoplasmic fraction of control NCTC 2544 keratinocytes with sodium deoxycholate greatly increased the NF- κ B DNA-binding activity in the cytosol, as compared with non-treated extracts. In contrast, the same treatment did not increase the NF- κ B DNA-binding activity of the cytoplasmic fraction from irradiated keratinocytes. These results strongly suggest that the decrease in NF- κ B DNA-binding activity in UV-A-irradiated cells is related to an alteration in the levels of NF- κ B subunits in the cytoplasm.

To analyse further the mechanism of the UV-A-mediated decrease in NF- κ B DNA-binding activity, we investigated the effect of UV-A on NF- κ B protein expression. Cytoplasmic, nuclear and total extracts prepared from NCTC 2544 keratinocytes irradiated or not with UV-A radiation were analysed by Western blots. As shown in Figure 4(B), the level of immunoreactive p65 in the total extract decreased upon UV-A irradiation. Figure 4(B) also shows a marked decrease in the levels of the p65 protein in the nuclear and cytoplasmic extracts from UV-A-irradiated cells as compared with sham-irradiated control keratinocytes. In the case of p50, we used two antibodies that recognize respectively the N-terminal (1141) and the C-terminal (1157) domains of this protein. As shown in Figure 4(B), the level of immunoreactive p50 was decreased in the total, cytoplasmic and nuclear fractions of irradiated cells. Overall, these results indicate that UV-A exposure specifically decreases the levels of the p65 and p50 NF- κ B subunits.

Effects of antioxidants on NF- κ B DNA-binding activity

Since UV-A exposure has been shown to induce membrane lipid peroxidation in fibroblasts and keratinocytes [23], we investigated the effects of lipophilic antioxidants such as vitamin E on the

Figure 4 UV-A-mediated decreases in the latent cytosolic NF- κ B DNA-binding activity, and in p50 and p65 protein levels

(A) Cytosolic extracts prepared from sham-irradiated (—) or UV-A-irradiated (+; 240 kJ/m²) cells were maintained for 15 min at 0 °C in the absence or presence of 0.8% sodium deoxycholate (DOC) and assayed for NF- κ B DNA binding. Identical results were obtained in two other experiments. (B) Cells were treated with UV-A. Cytoplasmic (C), nuclear (N) and total (T) fractions (10–30 μ g) prepared from cells were separated by SDS/PAGE and transferred to an Immobilon membrane. Immunoblotting was performed using 1:1000 dilutions of polyclonal antibodies raised against p65 (1226) or p50 (1141 and 1157) proteins. Results are representative of four independent experiments.

Figure 5 UV-A-mediated decrease in NF- κ B DNA binding is modulated by enhancers and quenchers of singlet oxygen, but not by vitamin E

NCTC cells were sham-irradiated (—) or UV-A-irradiated (+; 240 kJ/m²) in the presence or absence of sodium azide (NaN₃) or deuterium oxide (D₂O), or were pre-incubated for 24 h with or without vitamin E (Vit.E). Nuclear extracts were prepared 3 h after UV-A exposure. (A) TBARS were measured following cell treatment in an aliquot of the irradiated medium. C, control. (B) Nuclear extracts were assayed for NF- κ B DNA-binding activity as described in the Materials and methods section.

appearance of lipid peroxidation products (TBARS) and NF- κ B DNA-binding activity. Figure 5(A) shows that pretreatment of NCTC 2544 keratinocytes with vitamin E decreased by about 80 % the TBARS formation due to UV-A radiation. In contrast, as shown in Figure 5(B), this antioxidant did not significantly prevent the UV-A-mediated decrease in NF- κ B DNA-binding activity. The generation of singlet oxygen has been shown to play an important role in cellular responses to UV-A radiation [30,31]. Therefore, in the present study, reagents capable of quenching (sodium azide) or enhancing (²H₂O) singlet oxygen were tested for their capacity to affect the UV-A-mediated decrease in NF-

κ B DNA-binding activity. As shown in Figure 5(B), irradiation in the presence of sodium azide completely inhibited the decrease in NF- κ B DNA-binding activity induced by UV-A. In addition, exposure of NCTC 2544 keratinocytes to UV-A radiation in the presence of ²H₂O enhanced the effect of UV-A on NF- κ B DNA-binding activity. These observations suggest that singlet oxygen is involved in mediating the UV-A-induced decrease in NF- κ B DNA-binding activity.

DISCUSSION

The two main cell types represented in the skin, i.e. keratinocytes (epidermis) and fibroblasts (superficial dermis), are potential targets of UV-A radiation. It is noteworthy that only keratinocytes in the basal layer are able to divide, and thus play a major role in the renewal of superficial skin. From this point of view, dividing keratinocytes such as NCTC 2544 cells may be considered as a good experimental model for the study of the effects of UV-A on the basal layer of the epidermis. The present work was designed to examine the effect of UV-A-induced oxidative stress on NF- κ B DNA-binding and transcriptional activities in human NCTC 2544 keratinocytes. Several conclusions can be drawn from this work, as follows.

UV-A exposure induces a dose-dependent decrease in NF- κ B DNA-binding activity in human NCTC 2544 keratinocytes. A significant effect is observed at irradiation doses as low as 60 kJ/m². Furthermore, considerable inhibition (75–80 %) of NF- κ B DNA binding was obtained with the maximal UV-A dose used in this study (240 kJ/m²). As a positive control, we showed that the NF- κ B DNA-binding activity of the NCTC cell line can be greatly increased in the presence of well-known NF- κ B activators, consistent with previous studies in other cell lines. Interestingly, under identical conditions, the DNA-binding activity of the AP-1 transcription factor is induced by UV-A exposure [32]. This finding is indicative of the non-cytotoxicity of the UV-A dose used in our studies. It should be noted that the maximal UV-A dose that we used (240 kJ/m²) is equivalent to only one-third of the UV-A minimal erythral dose for a phototype I subject. We thus provide the first evidence that a moderate dose of UV-A may regulate the NF- κ B and AP-1 transcription factors in opposite directions.

Pretreatment of NCTC 2544 keratinocytes with UV-A impaired the activation of NF- κ B by inducers such as PMA, TNF α and UV-B, suggesting that UV-A affects the cellular elements necessary for NF- κ B activation. These findings were confirmed by immunofluorescence studies, which showed that the nuclear p65

staining obtained with PMA is lost if the keratinocytes are previously UV-A-irradiated (results not shown). Moreover, we show here that UV-B, unlike UV-A radiation, strongly induces NF- κ B DNA-binding activity in NCTC 2544 keratinocytes. Pretreatment of the keratinocytes with UV-A radiation inhibited the activation of NF- κ B induced by UV-B. These results support the proposal that UV-A and UV-B affect the cellular components differently. Since the solar UV radiation reaching the earth's surface is a mixture of UV-A and UV-B, these opposite responses may be a natural cellular defence required to balance the activity of NF- κ B. Hyperthermia has been shown to have a similar protective effect against UV-B irradiation in keratinocytes [33,34].

Our findings contradict the increased NF- κ B DNA-binding activity exhibited by UV-A-treated human fibroblasts reported by Vile et al. [35], and also differ from the results of Tobin et al. [36], which showed no effect of UV-A exposure on NF- κ B DNA binding in murine keratinocytes. These dissimilar results may be explained by the different irradiation conditions and different cell types. For example, many UV-A tables contain a small amount of UV-B which can interfere with or even mask the cellular responses mediated by UV-A, as a small dose of UV-B may trigger a large biological response compared with the effects of UV-A. Therefore, in order to study the effects of UV-A radiation on cell components, it is essential to remove this residual UV-B radiation by using appropriate filters that absorb it (see the Materials and methods section). Another point to be considered is that, in many studies, NF- κ B activity is mainly measured by EMSA gel assays. In this system, which is limited by the concentration of nuclear extracts, it is difficult to evaluate a decrease in NF- κ B DNA-binding activity if the basal activity is already low. In our case, we showed by EMSA that unstimulated NCTC cells have a significant basal level of NF- κ B DNA binding that can be stimulated or inhibited by different agents.

Conversely, our data are in agreement with studies demonstrating that various stress agents can inhibit NF- κ B DNA binding. Shumilla et al. [37] showed inhibition of NF- κ B binding by metals such as chromium, cadmium, mercury, zinc and arsenite. These authors suggested that the mechanism of this inhibition involves interactions with critical protein thiol groups present in the NF- κ B binding domain. Recently, Sekkai et al. [38] also demonstrated an inhibition of NF- κ B and HIV-1 long-terminal repeat transcriptional activation by inducible nitric oxide synthase 2 activity. This finding conflicts with others, and may be explained by the different flux of NO used in these experiments. Thus the modulation of NF- κ B activity by different stress agents appears to be complex, since it depends on the nature and the dose of the oxidant used, and also on the redox status of the cell [39].

The decrease in NF- κ B DNA binding reported above is associated with a partial inhibition of NF- κ B transcriptional activity after UV-A exposure of NCTC keratinocytes. Similar results were obtained in three other cell lines, even in cells expressing high levels of NF- κ B activity, which argues further for a general response to UV-A. Moreover, in agreement with these results, we show that the expression of I κ B α , which is closely associated with NF- κ B activity, is also decreased upon UV-A irradiation. Taken together, these results indicate that UV-A can affect the transcriptional activity of NF- κ B target genes and reflect a functional inhibition of the NF- κ B complexes by UV-A exposure.

The decrease in p50/p65 heterodimers observed in nuclear fractions from irradiated keratinocytes was associated with a marked decrease in NF- κ B cytosolic stores, as evaluated by EMSA analysis of detergent-disrupted cytosolic fractions. The

UV-A-induced decrease in NF- κ B DNA-binding activity is therefore I κ B-independent: deoxycholate failed to restore this binding activity. This finding suggests that the reduction in nuclear NF- κ B activity induced by UV-A may be, at least partially, the result of an alteration of the cytoplasmic NF- κ B complexes. Consistent with this hypothesis, we showed that the UV-A-mediated inhibition in NF- κ B activity is associated with a marked decrease in the levels of both the p65 and p50 proteins, as indicated by decreases in the levels of p50 and p65 immunoreactive proteins in the cytoplasm of these NCTC cells. Moreover, we found a similar result when we measured the levels of p50 and p65 proteins in nuclear extracts from UV-A-irradiated NCTC 2544 keratinocytes, which argues for a correlation between the initial signal beginning in the cytoplasm and the nuclear UV-A responses. In other words, the release of I κ B from the complex is not able to activate the transcription factor, because the NF- κ B subunits have been inactivated. These results suggest the occurrence of a protein degradation response following UV-A irradiation, and this is supported by the finding that, under identical conditions, UV-A exposure did not affect the expression of the p50 and p65 mRNAs (results not shown). However, the possibility cannot be excluded that the mechanism of UV-A-mediated NF- κ B inhibition also involves other cellular processes.

It has been demonstrated that UV-A irradiation triggers the appearance of reactive oxygen species and lipid peroxidation products. In the present study, we show that vitamin E effectively prevents UV-A-mediated lipid peroxidation formation, but has no significant effect on UV-A-mediated NF- κ B inhibition. This suggests that no correlation may be drawn between UV-A-induced peroxidative membrane damage and UV-A-mediated NF- κ B inhibition. It is noteworthy that vitamin E is localized inside the lipid phase of the cell membrane and can act as a radical scavenger in the membrane environment of the cell, but not in the cytosolic and nuclear compartments. It is possible that the reactive oxygen species generated by UV-A exposure affect, directly or indirectly, the NF- κ B complexes in the cytosol and therefore trigger their inactivation. Singlet oxygen has been shown to serve as a primary effector in many responses to UV-A [30,31]. No direct method exists to detect singlet oxygen in irradiated cells. Evidence for the involvement of singlet oxygen is therefore based on indirect methods, including the use of singlet oxygen quenchers and enhancers. In the present work, using this approach, we provide evidence that UV-A-generated singlet oxygen is involved in the UV-A-mediated decrease in NF- κ B activity.

The pattern of expression of NF- κ B and its effect on globular cellular processes have been well characterized in immune cells [40], but less is known about this transcription factor in skin cells. Recently, Seitz et al. [41] have demonstrated that NF- κ B activation in stratified epithelium is important for cellular growth arrest, in contrast with other tissues [42,43]. The present study provides clear evidence that UV-A radiation specifically decreases both NF- κ B DNA binding and transcriptional activities in human keratinocytes. The formation of singlet oxygen is likely to be the proximate cause of this effect. In the light of these observations, the bearing of the present results on the role played by UV-A in skin cell proliferation or apoptosis deserves further investigation.

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