Ultraviolet-A induces activation of AP-1 in cultured human keratinocytes

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Abstract UV-A irradiation induces a time-dependent activation of AP-1 in NCTC 2544 human keratinocytes. 4 h after irradiation, a 2–3-fold increase in AP-1 activity is observed in human keratinocytes and fibroblasts. Activation is still detectable 24 h later. The UV-A induced AP-1 binding complex is shown to contain c-Fos and c-Jun proteins. Lipophilic vitamin E impedes UV-A induced lipid peroxidation but does not prevent AP-1 activation which is inhibited by *N*-acetylcysteine, a hydrophilic antioxidant. This finding suggests that UV-A-dependent AP-1 activation is sensitive to the cellular redox state but is not related to membrane lipid peroxidation.

Key words: Ultraviolet A; Keratinocyte; Transcription factor AP-1; Antioxidant; Lipid peroxidation

1. Introduction

The ultraviolet spectral distribution of solar radiation at the Earth's surface includes short-wavelength UV-B light (290–320 nm) and long-wavelength UV-A light (320–400 nm). UV-B and UV-A have been shown to be involved in degenerative changes in the skin related to aging and cancer [1,2]. UV-A is more abundant (96% of UV radiation) and more penetrating than UV-B, making it possible for UV-A to play an important role in dermal and epidermal damage. Traditionally, many studies have been focused on UV-B and UV-C (e.g. wavelength in the range 100–290 nm), because these radiations can directly damage nucleic acids [3]. Much less attention has been paid to the potential contribution of solar UV-A which is expected to affect different cell components. Such damage is thought to result from a photodynamic action of UV-A, involving endogenous chromophores such as flavins or porphyrins [4], which produce reactive oxygen intermediates (ROI) in the cell. The ROI can damage cells via lipid peroxidation and alteration of proteins and nucleic acids [5]. Indeed, exposure of mammalian cells to UV-A has been shown to result in alterations of protein kinase C activity [6], activation of phospholipases A_2 and C [7], and lipid peroxidation [8]. Recently, we have demonstrated that UV-A decreases receptor-mediated processing of low-density lipoprotein [9] and of epidermal growth factor in human fibroblasts and keratinocytes [10]. Little is known about the effect of UV-A on factors that regulate gene expression but the induction of NF-κB DNA binding activity by UV-A was recently demonstrated in human fibroblasts [11]. Besides NF-κB, AP-1 is known as an oxidative stress-responsive transcription factor.

The transcription factor AP-1 is composed of members of the Jun and Fos families which bind to PMA response elements or AP-1 sites. The AP-1 couples extracellular signals such as growth factors and PMA to changes in the program of gene expression associated with growth, differentiation and cellular stress [12]. It has been shown that stimulation of AP-1 by growth factors involves a cascade of kinase activation [13]. Several pathways and mechanisms, including c-*fos* and c-*jun* induction and post-translational modification of their products, contribute to the induction of AP-1 activity. Recent results have demonstrated an increase in AP-1 binding activity in response to a wide range of stresses, including ionizing radiation, heat shock and UV-C radiation [14,15]. These findings prompted us to investigate whether the UV-A induced oxidative stress was able to modulate the activity of AP-1 binding sites and, if so, to examine the effect of antioxidants on this response. Here, we demonstrate a dose-dependent activation of AP-1 after UV-A exposure of NCTC 2544 human keratinocytes. Vitamin E, a specific membrane lipid antioxidant, does not prevent the action of UV-A on AP-1 activation while *N*-acetylcysteine (Nac), a glutathione precursor, impairs this response.

2. Materials and methods

2.1. Reagents

The double-stranded oligonucleotides containing the AP-1 sequence were synthesized on a 0.2 μmol scale by Eurogentec (Belgium). [γ-^{32}P]ATP was purchased from ICN (Orsay, France). T4 polynucleotide kinase was obtained from Boehringer Mannhein, (Meylan, France). Rabbit polyclonal antibodies against c-Fos and c-Jun subunits of AP-1 were provided by Santa Cruz Biotechnology, Inc. (CA, USA). The Oct-1 consensus oligonucleotide was synthesized by Promega (Charbonnières,France). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The NCTC 2544 human keratinocyte cell line [16] was purchased from ICN (Orsay, France) and the MRC5 human fetal lung fibroblasts was obtained from BioMérieux (Craponne, France). Cells were cultured at 37°C in Dulbecco's modified Eagle's Medium (DMEM, Gibco-BRL). supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Prior to UV-A irradiation, cells were growth-arrested at subconfluence by a 24 h incubation with serum-free DMEM supplemented with 0.1% bovine serum albumin.

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Abbreviations: UV, ultraviolet; AP-1, activator protein-1; Nac, *N*-acetylcysteine; PMA, phorbol 12-myristate 13-acetate; MDA, malondialdeyde; TBARS, thiobarbituric acid reactive substances; EMSA, electrophoresis mobility shift assay.

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2.3. UV-A irradiation

Ultraviolet-A radiation was carried out with a Vilber Lourmat table equipped with a TF-20L tube, emitting maximally at 365 nm, at a dose rate of 3 ± 0.2 mW/ cm^2. A piece of glass (4 mm thickness) was placed 20 mm above the table to absorb the majority of UV-B light (transmittance <0.01% at 310 nm). Before irradiation, cell monolayers were washed three times with PBS (phosphate buffer, saline) and covered in Hanks' salts. After irradiation with UVA doses as high as 190 kJ/m^2, cells were left for 1 h in the dark at 37°C. Then, 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.

2.4. TBARS measurement and effect of antioxidants

Lipid peroxidation end products (TBARS), including malondialdehyde (MDA), were measured after 1 h following cell irradiation. Measurements were performed on an aliquot of the supernatant of irradiated or sham-irradiated cells, as it has been previously shown that most of the TBARS produced after UV-A exposure are secreted by cells [8]. The samples were assayed for TBARS, using the fluorimetric method of Yagi [17], with minor technical modifications [8]. In some experiments, the effects of the antioxidants vitamin E and *N*-acetylcysteine were studied. *N*-Acetylcysteine solution was adjusted to pH 7.4 with 1 M NaOH. This agent was added to cultures 2 h prior to irradiation at 3×10^{-2} M and then reintroduced with Hank's salt following irradiation. It is interesting to note that *N*-acetylcysteine solution had no significant absorption in the UV-A range. In studies where vitamin E was utilised, this antioxidant was added to cultures 24 h prior to irradiation at 5×10^{-5} M with serum-free DMEM supplemented with 0.1% bovine serum albumin.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared as described by Dignam et al. [18] with slight modification. Briefly, cells were harvested, pelleted and resuspended in 100 μl of 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P-40 supplemented with the following protease inhibitors: 0.2 mM phenylmethylsulfonyl floride, 2 μM aprotinin, 1 μg/ml antipain, pepstatin, benzamidine, and leupepetin. After homogenization in a tight-fitting Dounce homogenizer, cell lysates were left on ice for 10 min and then centrifuged at $2000 \times g$ for 30 min. The nuclear pellet was then washed with buffer A without Nonidet-40, and resuspended in 50 μl of 20 mM HEPES, pH 7.9, 490 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 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 $13\,000 \times g$, for 15 min, the nuclear extracts (supernatants) were collected and frozen in aliquots 15 μl at −80°C.

2.6. Electrophoretic mobility shift assay (EMSA)

Either strand of the AP-1 duplex was ^{32}P-end labeled with T4 polynucleotide kinase and [γ-^{32}P]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 nuclear protein in 20 μl of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.2% Nonidet P-40, and 3 μg of poly(dI-dC). The solution contained 0.1 pmol of the end-labeled double-stranded AP-1 probe (20 000–40 000 cpm). Where indicated, the incubation mixture also contained a large excess of a double-stranded unlabeled oligonucleotide containing either the AP-1 (specific) or the OCT-1 (immunoglobulin enhancer octanucleotide factor-1, an unspecific double-stranded oligonucleotide of identical length) consensus sequence. The binding mixtures were electrophoresed on a 6% non-denaturing polyacrylamide gel in 180 mM Tris, 180 mM borate, 0.1 mM EDTA, pH 8.3, dried and analyzed with a phosphorimager instrument SP (Molecular Dynamics). For the characterization of the DNA binding proteins, nuclear proteins were incubated for 24 h at 4°C with specific antibodies directed against c-Jun or c-Fos before the binding reaction was initiated.

3. Results

3.1. Activation of AP-1 by UV-A

The AP-1 activity was measured at different times after UV-A exposure of NCTC 2544 human keratinocytes (Fig. 1). This activity was relatively low in controls (serum-starved cells), as shown by the weak intensity of the DNA-binding complex band in the case of sham-irradiated cells. Rapid activation of AP-1 binding sites was already detectable 1 h after irradiation at 190 kJ/m^2. 4 h after irradiation, the intensity of the DNA-binding complex band shown a maximal 3-fold increase as compared to sham-irradiated controls. Furthermore, 24 h after UV-A exposure, activation of AP-1 by UV-A was attenuated but was still higher than that of control cells. To approach the mechanism whereby UV-A induced AP-1, NCTC 2544 cells were treated before, during and after irradiation with the protein synthesis inhibitor cycloheximide. Interestingly, we observed that activation of AP-1 by UV-A measured 1 h after irradiation was preserved in the presence of cyloheximide (Fig. 1, last two lanes).

Fig. 2A shows the effect of increasing UV-A doses on AP-1 activity, measured 4 h after irradiation in human keratinocytes NCTC 2544. Significant activation of AP-1 DNA binding can be observed with low doses of UV-A (47.5 kJ/m^2). The extent of activation was dose-dependent, with a reproducible 2–3-fold enhancement at the maximal UV-A dose (190 kJ/m^2). Similarly, this UV-A dose increased the AP-1 activity by about 3-fold in human fibroblasts (MRC5 cell line, Fig. 2B).

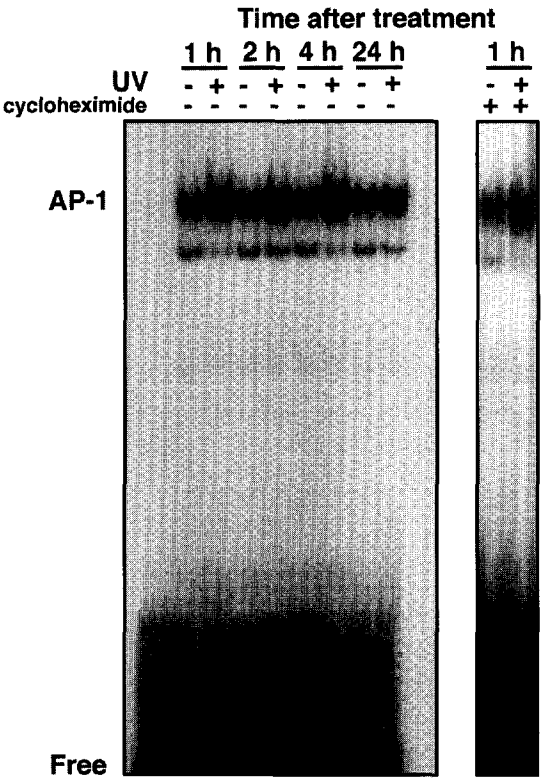

Fig. 1. Time course of AP-1 activation by UV-A. NCTC 2544 cells were treated with (+) or without (−) 190 kJ/m^2 dose of UV-A and then harvested at different time intervals after treatment. In the last two lanes, cells were incubated in the presence (+) of 10 μg/ml cycloheximide 2 h before irradiation. Following irradiation, the drug was reintroduced into the cultures. Nuclear extracts were prepared and assayed for AP-1 activity, as described in section 2. These data are representative of at least three independent experiments. The specific AP-1 DNA binding complex bands are indicated by 'AP-1' and the free probe bands are denoted by 'free'.

Table 1
Effect of vitamin E and *N*-acetylcysteine on UV-A induced TBARS production and AP-1 activity

	TBARS formation (nmol/mg cell protein)	AP-1 activity (%)
N	0.3 ± 0.015	100
I	1.3 ± 0.14	232 ± 26
I+vit E	0.4 ± 0.03	240 ± 17
I+Nac	1.1 ± 0.1	131 ± 16

Prior to irradiation, cells were incubated either for 24 h with 5×10^{-5} M vitamin E or for 2 h with 3×10^{-2} M Nac. Cells were irradiated with a dose of 190 kJ/m^2. TBARS were measured after 1 h of incubation at 37°C in the dark. Nuclear extracts were prepared 4 h after UV-A exposure. EMSA was performed as described under section 2. For TBARS, results are expressed as nmol MDA equivalents/mg of cell proteins. AP-1 activity is expressed as percentage of controls (N). N, unirradiated cells; I, irradiated cells; I+E, irradiated cells pre-treated with vitamin E; I+Nac, irradiated cells pre-treated with Nac.

3.2. *Characterization of the UV-A-induced AP-1-DNA complex*

The UV-A induced DNA-protein complex was characterized (figure 3) using antibodies directed against the c-Jun (lane 2) and c-Fos (lane 3) AP-1 subunits. Incubation of nuclear extracts with these antibodies markedly reduced the intensity of the DNA protein complex band indicating the presence of c-Jun and c-Fos in the UV-induced complexes. The specificity of the DNA-protein complex was further assessed by competition experiments (figure 3). The binding to the oligonucleotide was strongly inhibited in the presence of a large excess of unlabeled oligonucleotide containing the AP-1 binding site (lane 4). No competition was detectable upon addition of a large excess of non-specific double-stranded DNA containing the Oct-1 consensus sequence (lane 5).

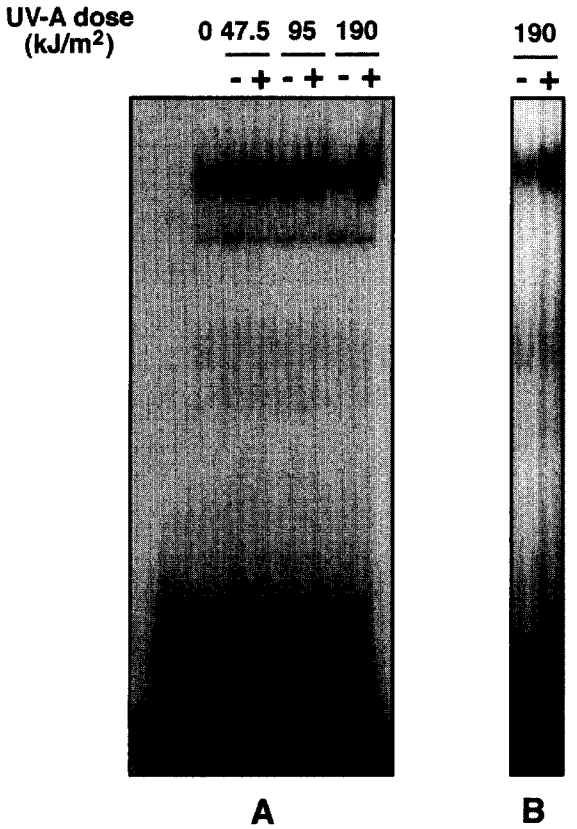

Fig. 2. Dependence of AP-1 activation on UV-A doses. (A) NCTC 2544 cells were treated with (+) or without (−) UV-A doses as indicated at the top of the figure and then left for 1 h at 37°C in the dark. Nuclear extracts were prepared 4 h after irradiation and assayed for AP-1 activity, as described under section 2. Data are from a typical experiment that was repeated three times. (B) MRC5 cells (human fibroblasts) were treated with (+) or without (−) UV-A at the maximum dose of 190 kJ/m^2, as indicated at the top of the figure, and then treated as NCTC 2544 cells.

3.3. *Effect of antioxidants on AP-1 activity*

Since UV-A exposure has been shown to induce membrane lipid peroxidation in fibroblasts and keratinocytes [8], we investigated the role of lipid peroxidation in UV-A induction of AP-1 activity. Therefore, the effects of a pre-treatment of cells with antioxidants such as the lipophilic vitamin E and the hydrophilic *N*-acetylcysteine were studied. Table 1 shows that, under our conditions, vitamin E efficiently reduced–by about 95%–the UVA-induced formation of lipid peroxidation products (TBARS). In contrast, this antioxidant did not prevent activation of AP-1 DNA binding activity by UV-A. On the other hand, *N*-acetylcysteine, which is readily taken up by cells and is rapidly converted to glutathione, did not impede lipid peroxidation but inhibited the UV-A activation of AP-1 (Table 1).

4. Discussion

The present work reports the effect of UV-A exposure on AP-1 binding activity in human keratinocytes which represent about 95% of all epidermal cells. Similar activation was obtained in human fibroblasts (figure 2B). Several conclusions can be drawn from this study.

Firstly, UV-A exposure induces a time-dependent activation of AP-1. This effect is already observed 1 h after UV-A exposure, is maximal after 4 h and is still detectable 24 h later. The mechanism whereby UV-A induces AP-1 activation is unknown but the finding that AP-1 activation occurs within the first hour of irradiation argues for a post-translational effect of UV-A on AP-1 activity. Moreover, treatment of cells before, during and after irradiation with cycloheximide, a protein synthesis inhibitor, maintains the UV-A activation of AP-1 measured 1 h after UV-A exposure, suggesting that this process did not require de novo protein synthesis at least over this time (see figure 1, right lanes). However, it cannot be excluded that the mechanism of activation of AP-1 at longer times such as 24 h after irradiation may involve more complex processes.

Secondly, UV-A exposure induces a dose-dependent increase in AP-1 binding activity. A significant effect is observed at a very low irradiation dose of 47.5 kJ/m^2. Furthermore, considerable activation of AP-1 is obtained at the maximal UV-A dose used in this study (190 kJ/m^2). Moreover, similar results are obtained with human fibroblasts (MRC5) suggesting that UV-A dependent activation of AP-1 is not a cell-type specific response. It is interesting to note that a UV-A dose of 190 kJ/m^2 is not cytotoxic towards these two types of cell lines

[19] and corresponds to only about 1/3 of the UV-A minimal erythemal dose for a phototype I subject (i.e., white caucasian).

Thirdly, vitamin E totally inhibits UV-A induced lipid peroxidation formation but does not prevent the increase in AP-1 activity. In addition, a significant activation of AP-1 is observed at low UV-A doses, where no TBARS are produced (data not shown). Together, these findings suggest that no correlation can be drawn between UV-A-induced peroxidative membrane damage and UV-A-enhanced AP-1 binding activity. Furthermore, we show here that, in contrast to vitamin E, *N*-acetylcysteine inhibits UV-A activation of AP-1 without affecting lipid peroxidation. Such a difference in the effect of these two antioxidants may be explained by their distinct mechanisms of action. Indeed, the hydrophobic vitamin E is localized inside the lipid phase of the cell membrane and can only act as a radical scavenger in the membrane environment of the cell [20]. On the other hand, the hydrophilic *N*-acetylcysteine, precursor of glutathione, can pass through the cell membrane and play a role in the regulation of the intracellular redox state of the cell by donation of an electron or a hydrogen atom from the sulphur moiety [21]. It is possible that UV-A perturbs the redox states of cellular proteins. This is consistent with the assumption that endogenous glutathione protects human skin fibroblasts against the cytotoxic effect of UV-A [22]. These results are also in accordance with previous findings which reported the UV radiation protecting efficacy of cysteine derivatives such as *N*-acetylcysteine [23,24]. It is interesting to note that the modification of cysteine residues on the DNA binding domain of c-Jun and c-Fos can modulate AP-1 activity [25]. In addition, it has been reported that Nac prevents the UV-C dependent stimulation of *src* tyrosine kinase activity which mediates the UV-C induced activation of AP-1 [26,27]. Thus, the redox sensitive kinase cascades involved in AP-1 signaling may be targets of UV-A radiation.

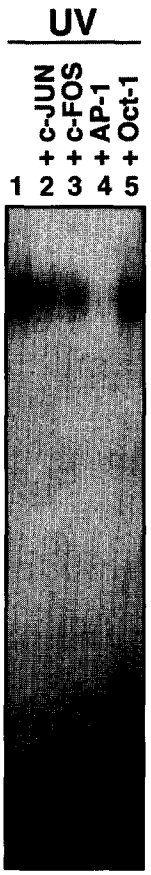

Fig. 3. Characterization of UV-A induced AP-1-DNA complexes. Nuclear extracts prepared from NCTC 2544 cells irradiated with 190 kJ/m2 UV-A were assayed for AP-1 binding activity with or without 2 mg of anti-c-Jun (lane 2) or anti-c-Fos (lane 3) antibodies, or in the presence of a 300-fold excess of an unlabeled oligonucleotide containing AP-1 sites (lane 4) or a 100-fold excess of an unlabeled oligonucleotide containing the Oct-1 consensus sequence (lane 5).

In conclusion, our study provides the first evidence that UV-A radiation induces AP-1 activation in human cells. This response is sensitive to a perturbation of the cellular redox equilibrium. It is known that many promoters that are active in terminally differentiating epidermal cells contain functionally important AP-1 sites [28]. In this regard, the protective or degenerative role played by the UV-A induced activation of AP-1 in skin cells deserves further investigation.

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