Singlet oxygen mediates the activation of JNK by UVA radiation in human skin fibroblasts

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Abstract Ultraviolet A (UVA: 320–400 nm) radiation activates c-Jun–N-terminal kinase (JNK 2) in human skin fibroblasts. Exposure of cells to UVA (300 kJ/m^2) led to a 5-fold induction of JNK-activity which was significantly increased in the presence of D_2O, an enhancer of the lifetime of singlet oxygen. Sodium azide, a quencher of singlet oxygen, abolished the activation of JNK. A hydroxyl radical scavenger, mannitol, had no effect. Furthermore, photochemically produced singlet oxygen (Rose Bengal plus white light) was found to induce JNK activity. This was enhanced by D_2O and inhibited by azide. Thus, singlet oxygen activates and mediates the UVA-induced activation of JNK.

Key words: Singlet oxygen; c-Jun–amino-terminal kinase; Human skin fibroblast

1. Introduction

Singlet molecular oxygen ($^1O_2$) can be produced in biological systems by photoexcitation (Type II reactions) upon exposure of endogenous photosensitisers, such as flavins, quinones or porphyrins to ultraviolet A (UVA) [1], or by chemiexcitation [2,3]. Apart from being involved in the damaging effects of UVA radiation (320–400 nm) on cellular systems [4,5], $^1O_2$ modulates gene expression and herein mimics UVA radiation: Heme oxygenase-1 (HO-1) [6,7], interstitial collagenase (matrix metalloproteinase-1) [8,9], and intercellular adhesion molecule-1 (ICAM-1) [10] have been demonstrated to be induced by UVA and singlet oxygen that was produced either chemically by the thermodecomposition of $NDPO_2$ (the 1,4-endoperoxide of 3,3′-(1,4-naphthylidene) dipropionate) or photochemically.

Regarding the UVA or $^1O_2$-mediated signalling pathways leading to gene expression, the induced expression of ICAM-1 is mediated by activated transcription factor AP-2, but not AP-1 or NF-κB, in human keratinocytes [10]. Interrelated autocrine loops with the interleukins IL-1 and IL-6 being involved were made responsible for the induction of collagenase in human skin fibroblasts [11]. Additionally, NF-κB was recently shown to be activated in fibroblasts upon UVA treatment [12].

It is well established that short-wavelength ultraviolet radiation (UVC or UVB) induces the so-called UV response comprising the induction of immediate-early genes such as c-jun and c-fos [13]. The induced expression of these genes follows the activation of transcription factors that are phosphorylated by members of the MAPK family: upon activation by dual phosphorylation of a Thr*–Pro–Tyr* motif, c-Jun–N-terminal kinases (JNK; also termed stress-activated protein kinases, SAPK [14]), phosphorylate c-Jun [15,16], activating transcription factor-2 (ATF-2) [17], and Elk-1 [18]. JNKs have been demonstrated to be activated by UVC (< 280 nm) [16,19] and UVB (280–320 nm) [[20], P. Brenneisen et al., unpublished results] irradiation in a variety of cells, a possible exception being keratinocytes where JNKs seem to respond to UVC but not UVB [19].

Here we report the activation of c-Jun–N-terminal kinase 2 by UVA radiation which is the major part of the UV region of the solar spectrum that reaches the earth and by singlet oxygen which seems to mediate the UVA effects.

2. Materials and methods

2.1. Cell culture and cell viability assays

Human skin fibroblasts from foreskin biopsies were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (0.02 g/l), penicillin (20 000 IU/l), and ascorbate (50 mg/l). Cell viabilities were determined by measuring the ability of cells to produce a blue-colored formazan from the tetrazolium salt MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide).

2.2. Singlet oxygen and UVA sources

Singlet oxygen was produced by irradiating solutions containing varying concentrations of Rose Bengal (RB) for 10 min with a commercially available 500 W lamp from a fixed distance of 66 cm. UVA irradiations were performed at intensities of 42–44 mW/cm^2 for approximately 12 min (yielding a dose of 300 kJ/m^2) using a UVA700 illuminator from Waldmann (Villingen, Germany). For treatments in the presence of deuterium oxide (90%), 10× concentrated PBS was diluted in D_2O (99.9%; Sigma, Munich, Germany).

2.3. Determination of singlet oxygen

The production of singlet oxygen from RB+light was determined by the amount of endoperoxide ($EASO_2$) formed from anthracene-9,10-diyldiethyl disulfate (EAS) which was incubated with the irradiated RB solutions. $EASO_2$ was then assayed by reversed-phase HPLC [21]. Furthermore, the bleaching of 4-nitroso-*N*,*N*-dimethylaniline (RNO) was used to estimate $^1O_2$ production by RB+light [22,23].

2.4. Immunocomplex kinase assay

Skin fibroblasts were grown to approximately 70–80% confluency in 60 mm dishes, washed twice with phosphate-buffered saline (PBS) and incubated with serum-free medium for an additional 2 days; the serum-free medium was renewed once. Following the removal of medium and a wash step with PBS, the serum-starved cells were irradiated in PBS or in PBS containing additives. After irradiation, the cells were covered with serum-free medium, incubated at 37°C for 30 min, washed with PBS of room temperature and lysed with 300 μl of ice-cold RIPA-buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% (w/v) Nonidet P-40 (Sigma, St. Louis, MO), 0.5% (w/v) sodium desoxycholate, 0.1% (w/v) SDS, 0.2 mM Na_3VO_4,

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Abbreviations: JNK, c-Jun–N-terminal kinase; UVA, ultraviolet A radiation (320–400 nm); RB, Rose Bengal

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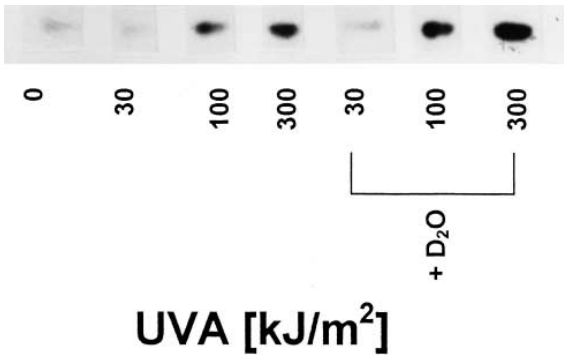

Fig. 1. Induction of c-Jun–N-terminal kinase activity (as assayed via phosphorylation of GST-cJun(1–79)) by various doses of UVA irradiation of human skin fibroblasts in the presence and absence of deuterium oxide.

0.8 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin). The lysed cells were scraped off and centrifuged. The protein content was determined using a commercially available protein assay (BioRad DC-Protein Assay). C-Jun–N-terminal kinase 2 (JNK 2) was immunoprecipitated for 2–3 h on ice from cell lysates (50 μg of protein in a total volume of 200 μl RIPA lysis buffer) using 10 μl of an anti-SAPK 2 antiserum (rabbit, diluted 1:10 in distilled water) which was a generous gift from Dr. Peter E. Shaw (Dept. of Biochemistry, University of Nottingham, UK).

Immune complexes were collected at 4°C overnight with 30 μl of Protein A Sepharose 6MB (Pharmacia, Freiburg, Germany) pre-equilibrated in RIPA lysis buffer. The pelleted immune complex/Protein A conglomerate was washed with both ice-cold RIPA buffer and kinase buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT) 3 times each.

Kinase buffer (35 μl) was added to the pellets, followed by 5 μl of 150 μM ATP, 6 μl (6 μg) of GST-cJun(1–79) (Alexis, Grünberg, Germany) [15], and 5 μl (5 μCi) of [γ^{32}P]ATP (5 Ci/μmol). The mixtures were incubated at 37°C for 25 min and repeatedly vortexed during this incubation time.

The reaction was stopped by the addition of gel loading buffer and denaturation for 5 min at 95°C. The samples were centrifuged for 2 min, and the supernatants analysed by electrophoresis on a 15% SDS-polyacrylamide gel. The phosphorylated JNK substrate (GST-cJun(1–79); 37 kDa) was identified by autoradiography and quantified by PhosphorImager™ analysis.

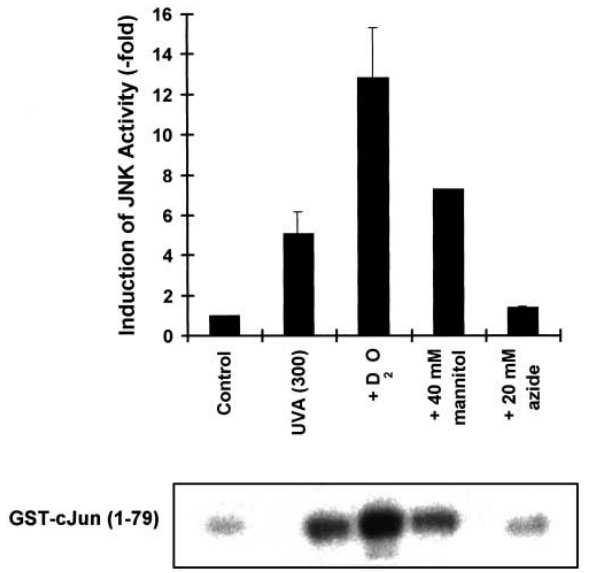

Fig. 2. Activation of JNK by UVA, enhancement by deuterium oxide and inhibition by azide. Values are means ± SEM (n = 3–6); data for mannitol: mean value of two experiments.

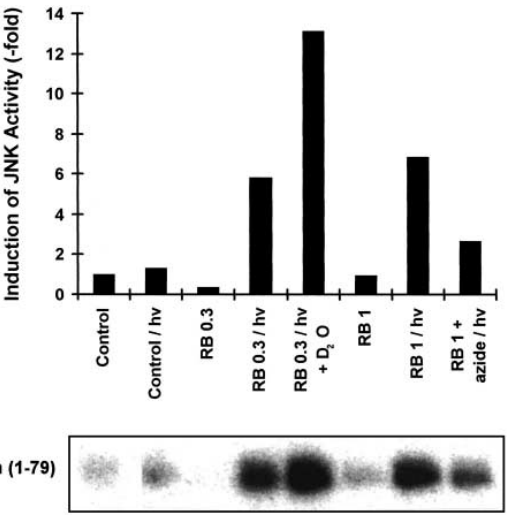

Fig. 3. Activation of JNK by singlet oxygen generated by RB+light; effects of deuterium oxide and sodium azide. Data are mean values of two experiments.

3. Results and discussion

C-Jun–N-terminal kinase (JNK 2) activity was enhanced by UVA irradiation (Fig. 1). A pronounced effect was observed at a dose of 100 kJ/m^2 (3-fold activation of JNK) and at 300 kJ/m^2 (5-fold activation), respectively (Figs. 1 and 2). The activation of JNK was substantially increased in the presence of deuterium oxide (≈13-fold), an enhancer of the lifetime of singlet oxygen. Furthermore, quenchers of singlet oxygen, such as azide (at 10 mM) and imidazole (at 20 mM, not shown), inhibited the increase in JNK activity induced by UVA irradiation (Fig. 2). Because hydroxyl radicals can be produced upon UVA irradiation in a Type I photodynamic reaction, the effect of the hydroxyl radical scavenger mannitol was tested; at 40 mM it had no effect. To test whether singlet oxygen generated in a model system is capable of inducing JNK activity, we used a known photosensitising system, RB plus light. Under the given conditions, singlet oxygen was produced at a rate of 22 μM/min and 103 μM/min upon irradiation of 0.3 μM and 1 μM RB, respectively. JNK activity was markedly enhanced by RB+light (≈5–6-fold; Fig. 3).

Table 1
Modulation of JNK activation after fibroblast exposure to UVA or RB+light by $^1O_2$ enhancer and quencher

Additive	JNK activity[a]	
	UVA	RB+Light
None	1	1
D_2O (90%)	2.6 ± 0.3	2.1
N_3^- [b]	0.3 ± 0.1	0.4
Mannitol (40 mM)	1.1	n.d.

n.d. = not determined.
[a]JNK activities after UVA irradiation (300 kJ/m^2) or exposure to RB (0.3 μM) + light of fibroblasts were set to 1. Values are given as means ± SEM (n = 3–6) or means of two experiments (mannitol and RB + light).
[b]Azide concentrations were 20 mM (UVA) or 50 mM (RB + light); RB concentration in the azide inhibition experiment was 1 μM.

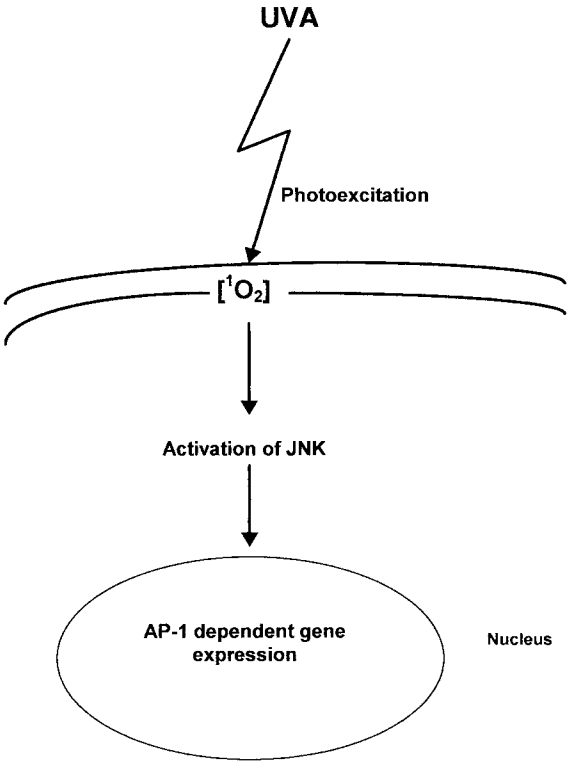

Fig. 4. Proposed scheme for the action of UVA via singlet oxygen affecting gene expression.

Again, D_2O acted as an enhancer of JNK stimulation ($\approx$13-fold activation) and azide (50 mM) inhibited by 55%. These observations support the hypothesis of singlet oxygen involvement in the activation of JNK in human dermal fibroblasts by UVA. The modulation of UVA-induced JNK activation by D_2O, azide and mannitol is documented in Table 1.

The correlation between UVA and singlet oxygen has already been made for the induced synthesis of both mRNA and protein of heme oxygenase-1 [6,7], collagenase (MMP-1) [8,9] and ICAM-1 [10]. The effects of UVA irradiation have been attributed to $^1O_2$ and can be simulated employing chemical and photochemical singlet oxygen sources. And vice versa, as various conditions of oxidative nature, such as the induction of oxidative metabolism in the liver of mice by injection of carbon tetrachloride [24], are now known to activate JNKs, we here specify singlet oxygen as one of the causative oxidative species.

It seems that singlet oxygen discriminatingly activates JNK but not ERK 1 or ERK 2, as RB+light could not be shown to effect a phosphorylation-induced shift in electrophoretic mobility of ERK 1 or 2 [F. Schliess et al., unpublished work].

Finally, the activation of JNKs by singlet oxygen and upon UVA irradiation suggests a role of transcription factor AP-1 [16] in the intracellular signalling processes induced in skin fibroblasts; in fact, the activation of AP-1 in human keratinocytes and fibroblasts after UVA irradiation has been documented [25]; a scheme outlining the proposed action of UVA and $^1O_2$ is shown in Fig. 4.

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