Singlet oxygen is an early intermediate in cytokine-dependent ultraviolet-A induction of interstitial collagenase in human dermal fibroblasts in vitro

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Abstract Ultraviolet (UV) A irradiation of human dermal fibroblasts elicits an increase in specific mRNA amounts and bioactivities of the cytokines IL-1α, IL-1β, and IL-6. These effects are enhanced in deuterium oxide-based medium and are diminished in the presence of non-toxic concentrations of sodium azide. Furthermore, generating singlet oxygen outside the cells by irradiation of rose bengal-coated resin particles with visible light ($\lambda > 450$ nm) results in the induction of interstitial collagenase, IL-1 and IL-6, similar to the response observed with UVA irradiation. These observations suggest that singlet oxygen is an early intermediate in the signaling pathway of IL-1 and IL-6 mediating UVA induction of interstitial collagenase (E.C. 3.4.24.7). Furthermore, singlet oxygen appears to initiate this complex UV response at the cell membrane.

Key words: Singlet oxygen; UV response; Cytokines; Connective tissue; Collagenase

1. Introduction

Skin is always in contact with oxygen and is increasingly exposed to ultraviolet (UV) radiation. Sunbathing for cosmetic reasons, therapeutic UV irradiation and stratospheric ozone depletion [1] increase the risk of photooxidative damage to the skin. Premature aging of skin secondary to chronic exposure to UV is mainly due to qualitative and quantitative alterations of the dermal extracellular matrix [2], resulting in increased wrinkle formation and impaired wound healing of the skin. Interstitial collagens, the major structural components of the dermis, have been found to be diminished particularly in actinically damaged skin [3,4]. In contrast to UVB and UVC, UVA irradiation is known to reach the reticular dermis making fibroblasts an accessible target [5]. Recent work has shown that UV irradiation substantially affects the coordinated regulation of various matrix-degrading metalloproteinases and their inhibitor, tissue inhibitor of metalloproteinases, TIMP-1 [6–10]. The imbalanced induction of collagenase is at least in part mediated by the proinflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) [11,12]. UVA irradiation alone and in conjunction with cellular photosensitizers has the potential of generating singlet oxygen and other reactive oxygen species [13–15]. In fact, singlet oxygen has been shown to be involved in the downstream signaling of the UVA response leading to collagenase induction [16].

However, it is still unresolved where UV-generated singlet oxygen may fit into the signaling cascade of this complex cellular UV response. Here, we show that generation of singlet oxygen is an early event at the cell membrane that precedes the induction of interrelated autocrine loops of IL-1 and IL-6, ultimately leading to tissue degradation in photoaging. The identification of singlet oxygen as an early event in the downstream signaling of the UV response is particularly important and promising for the design and development of UV-protective agents.

2. Materials and methods

2.1. Reagents

Deuterium oxide D_2O (99.9%) was from Aldrich (Steinheim, FRG), sodium azide from Merck (Darmstadt, FRG). The endoperoxide of the disodium salt of 3,3′-(1,4 naphthylidene) dipropionate ($NDPO_2$) was prepared as described [17]. Experiments were carried out with the compounds at non-toxic concentrations. Trizol was from Life Technologies (Eggenstein, FRG). Rose bengal was from Sigma (Deisenhofen, FRG), the basic anionic exchange resin Amberlite CG-400E was from Serva (Heidelberg, FRG). Rose bengal immobilized on amino agarose was from Molecular Probes (Eugene, OR).

2.2. Cell culture

Fibroblast cultures were established by outgrowth from foreskin biopsies of healthy human donors [18]. Cells were used at passage 5 to 10. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Flow, Meckenheim, FRG), supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS), and grown on plastic Petri dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.3. Cytotoxicity assay

Viability was checked 24 h after irradiation or incubation the cells with $NDPO_2$ or quenchers and enhancers, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [19].

2.4. Singlet oxygen generation

Singlet oxygen was generated by the thermal decomposition of the endoperoxide of 3,3′-(1,4-naphthylidene) dipropionate ($NDPO_2$) yielding 3,3′-(1,4-naphthylidene) dipropionate (NDP) [17]. Incubations were performed in PBS after the cells were washed free of medium and were terminated at 30 min. The rate of $^1O_2$ generation was monitored by the formation of NDP [17]; 15 min after addition of 1 mM $NDPO_2$ the rate of $^1O_2$ generation was 3 μM/min.

Furthermore, rose bengal was attached to a basic anionic exchange resin particles (Amberlite), using non-sedimenting particles [20] or rose bengal immobilized on amino agarose. Cells were irradiated for 3 min at a distance of 50 cm with a 150 W light source in the presence of rose bengal-coated resin particles. A cutoff filter was used to eliminate light at $\lambda < 450$ nm. At these conditions the rate of $^1O_2$ generation was 6 μM/min.

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2.5. UVA irradiation

Prior to UVA irradiation, fibroblast monolayers were washed twice with PBS. The cells were irradiated at a distance of 40 cm by a high intensity UV source emitting light of wavelengths in the 340–450 nm range (UVASUN3000 equipped with UVASUN safety filters; Mutzhas, Munich, FRG) [21]. Fluences (200 kJ/m^2) were determined with a combined UVA/UVB ultraviolet meter (Centra UV dosimeter, Osram, Munich, FRG) [22]. During irradiation the cells were incubated with PBS. After irradiation fresh medium with 10% fetal calf serum (FCS) was added, and cells were incubated until used for RNA extraction or bioactivity measurement.

2.6. Bioactivity of cytokines

Bioactivities of IL-1 and IL-6 were determined in the supernatants of fibroblast monolayers at different time points post irradiation by means of established proliferation assays (murine plasmacytoma cell line B9; murine T-cell line D10N4M) [23,24] as described [12].

2.7. RNA extraction and Northern blot analysis

Total RNA was extracted from the cells with Trizol according to the manufacturer's recommendations (Life Technologies, Eggenstein, FRG). For Northern blot analysis, 4 μg of total RNA were separated by denaturing agarose gel electrophoresis. RNA was blotted onto Genescreen membranes (Dupont NEN, Bad Homburg, FRG). ^{32}P-oligolabeled inserts with deoxycytidine-5′-[^{32}P]triphosphate (ICN, Meckenheim, FRG) with the Megaprime DNA labeling system (Amersham, Braunschweig, FRG) of the following cDNA clones were used for hybridization experiments: IL-1α (2000-bp *Pst*I/*Hin*cII fragment) [25], IL-1β (600-bp *Sma*I/*Bam*HI fragment) [26], IL-6 (1120-bp *Eco*RI fragment) [27], interstitial collagenase (MMP-1) (920-bp *Hin*dIII/*Sma*I fragment) [28], β-actin (450-bp *Eco*RI/*Bam*HI fragment) [29]. Hybridization experiments were performed as described elsewhere [12].

3. Results and discussion

Exposure of cultured human dermal fibroblasts to singlet oxygen generated from $NDPO_2$ or UVA irradiation resulted in an increase in specific IL-1 and IL-6 mRNA levels at 12 h after incubation (Table 1). The induction after exposure of fibroblasts to 1 mM $NDPO_2$ was equivalent to that observed with 200 kJ/m^2 UVA showing a similar time course. The increase in mRNA due to exposure of cells to UVA in D_2O-based PBS was about 1.5-fold that in normal PBS, attributable to the longer halflife of singlet oxygen in D_2O (Table 1).

Table 1
Induction of IL-1 and IL-6 mRNA after exposure to singlet oxygen, or UVA is modulated by $^1O_2$ enhancer and quencher

Additions	Fold increase		
	IL-1α	IL-1β	IL-6
Singlet oxygen			
$NDPO_2$ (1 mM)			
None	1.7	1.4	1.8
D_2O (90%)	2.6	2.3	2.5
Azide (5 mM)	1.3	1.1	1.3
Rose bengal[a]/light			
None	n.d.	2.5	3.3
D_2O (90%)	n.d.	5.0	4.1
Azide (5 mM)	n.d.	1.8	2.5
UVA (200 kJ/m^2)			
None	2.0	1.8	3.5
D_2O (90%)	3.6	3.2	5.0
Azide (5 mM)	1.5	1.3	2.0

Data are from densitometric analysis of Northern blots and represent fold-increase over untreated control. Densitometric data were standardized to β-actin levels (compare Fig. 2). Data are mean values of two experiments. n.d., not determined. Rose bengal[a] = rose bengal immobilized on agarose beads and irradiated with visible light as described in Section 2.

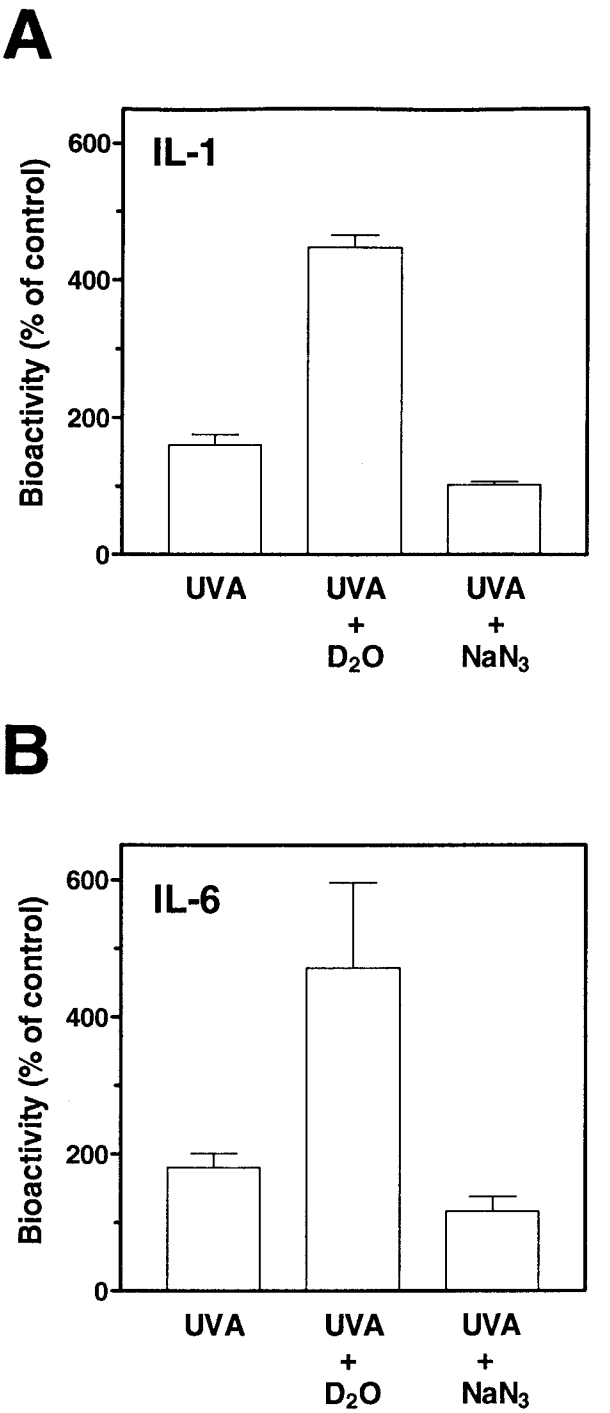

Fig. 1. Induction of IL-1 and IL-6 bioactivity in the supernatant of UVA irradiated fibroblasts is modulated by singlet oxygen enhancer and quencher. The bioactivity of IL-1 (A) and IL-6 (B) in supernatants of fibroblast monolayer cultures was evaluated 24 h after UVA irradiation using the bioactivity assays as mentioned in Section 2. The effect of singlet oxygen was modulated by D_2O based PBS and with 5 mM NaN_3. The cytokine bioactivity in mock-irradiated controls was set to 100%. Data are given as means ± S.D. ($n = 3$).

Sodium azide (5 mM), a potent quencher of $^1O_2$, significantly diminished the increase in IL-1 and IL-6 mRNA steady state levels upon exposure of cells to UVA. When supernatants from cultures of non-irradiated fibroblasts were tested for bioactive IL-1 or IL-6, respectively, constitutive cytokine ac-

tivity was detected in the absence of added stimuli. Following UVA irradiation, an up to two-fold increase in IL-1 (Fig. 1A) and IL-6 (Fig. 1B) bioactivities in the supernatants was detected at 24 h post irradiation. This increase in cytokine activity was even more enhanced in D_2O-based PBS and was nearly absent by adding the $^1O_2$ quencher sodium azide (5 mM) (Fig. 1). The apparently high concentration of azide was non-toxic, as assayed with MTT, in agreement with the observation that fibroblasts can tolerate even 100 mM azide for 2 h [30].

These observations provide evidence that UVA-generated singlet oxygen precedes and is, at least in part, responsible for the synthesis and release of bioactive IL-1 and IL-6. These cytokines have previously been shown by antisense strategies to mediate the induction of interstitial collagenase following UVA irradiation [11]. Singlet oxygen by itself is able to induce the synthesis of interstitial collagenase [16,31]. Thus UVA-generated singlet oxygen is identified as an early intermediate in the signaling cascade leading to the breakdown of connective tissue, and may represent a particularly attractive target for development of novel UV-protective agents.

In order to further address the question whether initial events of the UV-response occur at the cell membrane as suggested by Devary et al [32] or within the nucleus [33], we have used a system [20,34] generating singlet oxygen outside the cell. Interestingly, upon irradiation of rose bengal coated exchange resin particles with visible light (> 450 nm), a 7-fold induction of interstitial collagenase mRNA was observed

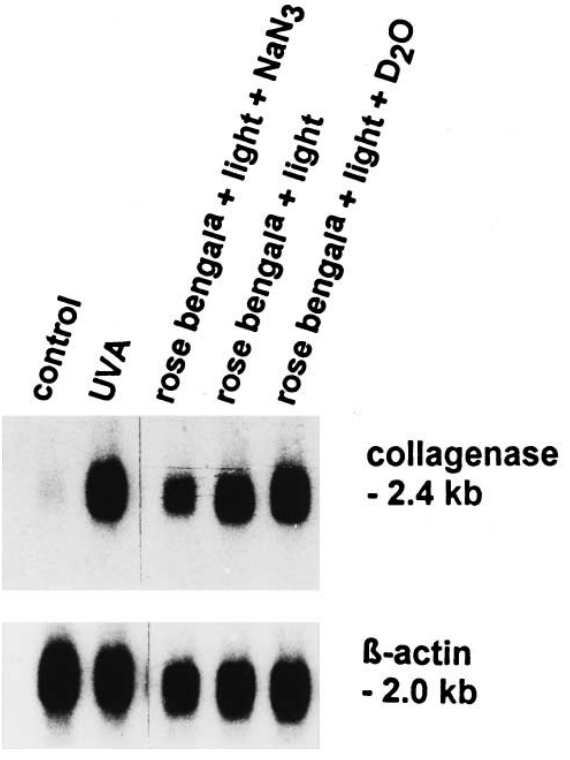

Fig. 2. Interstitial collagenase mRNA induction upon extracellular generation of singlet oxygen. Total RNA from fibroblast monolayer cultures was isolated at 24 h after treatment of the cells. The RNA was fractionated under denaturing conditions, blotted onto Genescreen membranes, and hybridized with specific cDNA probes for collagenase and β-actin. [a] = rose bengal attached to Amberlite CG-400E.

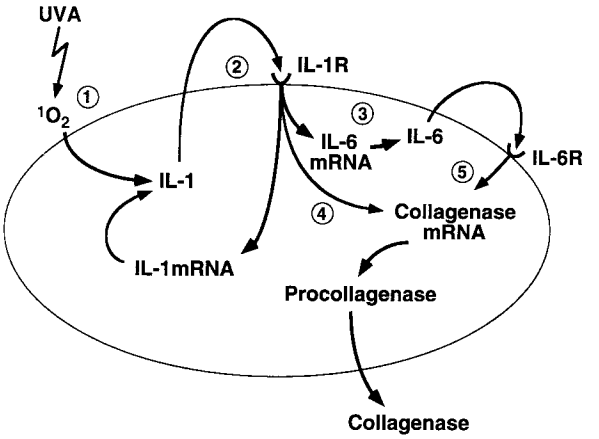

Fig. 3. Sequence of events upon UVA irradiation of dermal fibroblasts. The UVA induction of interstitial collagenase is mediated by singlet oxygen and interrelated autocrine loops of IL-1 and IL-6. The sequence of events is schematically delineated by encircled numbers. IL-1R = IL-1 receptor, IL-6R = IL-6 receptor.

(Fig. 2). The induction of collagenase was modulated by deuterium oxide and sodium azide in a similar way as seen for singlet oxygen generated by $NDPO_2$ [16,31]. Additionally, a significant induction in the mRNA steady state levels of IL-1 and IL-6 was observed upon irradiation of rose bengal immobilized to amino agarose with visible light. Likewise the induction of IL-1 and IL-6 was modulated by deuterium oxide and sodium azide in the expected fashion (Table 1).

These data support the concept that the cellular response to singlet oxygen may be initiated at the cell membrane possibly involving Src tyrosine kinases, HaRas small guanosine triphosphate binding proteins [32] or intermediates of lipid peroxidation ([35] and Brenneisen, unpublished results). In fact, IL-6 secretion by human fibroblasts after stimulation with IL-1 is inhibited by the chain-breaking antioxidant alpha-tocopherol [36] that also reacts with singlet oxygen [37].

In summary, we have identified singlet oxygen as an early intermediate in the UVA signaling pathway of interstitial collagenase induction, preceding synthesis of the proinflammatory cytokines IL-1 and IL-6 (see the scheme in Fig. 3).

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