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Singlet oxygen induces collagenase expression in human skin fibroblasts

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Singlet oxygen generated in a dark reaction by thermodissociation of an endoperoxide ($NDPO_2$) elicits an increase in mRNA of interstitial collagenase (MMP-1) in cultured human fibroblasts. The effect is enhanced in deuterium oxide-based medium and is abolished in the presence of non-toxic doses of sodium azide. In contrast, the mRNA level of the tissue inhibitor of metalloproteinases (TIMP-1) remains unaltered under these experimental conditions. These observations support the suggestion that an unbalanced synthesis of collagenase and TIMP reported to occur following UV-A irradiation or during inflammatory conditions may be mediated by singlet oxygen.

Singlet oxygen; Collagenase mRNA; TIMP mRNA; Connective tissue

1. INTRODUCTION

The integrity of the extracellular matrix responsible for proper organ function depends on the coordinated regulation of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). Fibroblast-derived collagenase (MMP-1) cleaves α-chains of interstitial collagen I and III and, in case of dysregulation, causes severe damage of the connective tissue. An unbalanced synthesis of collagenase and its counteracting inhibitor, TIMP-1, has been found in human fibroblasts in vitro and in vivo following UV-A irradiation and in inflammatory joint diseases such as arthritis [1–5]. Biological effects of UV-A irradiation can be mediated by electron transfer (Type I) or by an indirect pathway involving singlet oxygen (Type II) [6] (for review see [6a]).

In addition, singlet oxygen can be generated in biological systems, for example in skin, by photochemical reactions triggered by certain drugs or porphyrins, or by dark reactions, catalyzed by lipoxygenase, chloroperoxidase and lactoperoxidase [7–10], probably occurring during inflammation. The biological consequences of exposure to singlet oxygen include mutagenicity and genotoxicity [11,12]. Various genes have been described to be activated upon exposure of cells to singlet oxygen. For example, singlet oxygen can activate the HIV long terminal repeat integrated into cellular DNA of epithelial cells [13]. Interestingly, UV light has also been described to activate HIV gene expression [14]. Oxidative stress mediated by porphyrin photosensitization stimulates the expression of proteins of the heat-shock and glucose-regulated families, as well as of the heme oxygenase stress protein [15].

Even though the generation of singlet oxygen and the induction of collagenase are thought to occur both during inflammation and following UV-A irradiation, a direct relationship has not yet been established. We have investigated the role of singlet oxygen in the regulation of fibroblast collagenase and TIMP mRNAs by $^1O_2$ generated chemically from thermal dissociation of the endoperoxide of 3,3′-(1,4-naphthylidene) dipropionate ($NDPO_2$).

2. MATERIALS AND METHODS

2.1. *Reagents*

Deuterium oxide (99.9%) was from Aldrich (Steinheim, Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (Deisenhofen, Germany), sodium azide from Merck (Darmstadt, Germany). The endoperoxide of the disodium salt of 3,3′-(1,4-naphthylidene)dipropionate ($NDPO_2$) was prepared as described [16]. Preliminary experiments were carried out with the employed compounds to determine non-toxic concentrations.

2.2. *Cell culture*

Fibroblast cultures were established by outgrowth from skin biopsies of healthy human donors [17]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow, Meckenheim, Germany) supplemented with sodium ascorbate (0.25 mM), 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. Experiments were performed between passages 5 and 10.

2.3. *RNA extraction and Northern blot analysis*

Total RNA was isolated from cells following established procedures [18]. Briefly, fibroblast monolayer cultures were washed with PBS, denatured in 5 M guanidinium isothiocyanate and separated over a cesium chloride cushion at 35,000 rpm for 18 h.

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The pellet was washed in 70% ethanol, dried and dissolved in 20–100 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The amount of total RNA was determined photometrically at 260 nm. For Northern blot analysis, 2–8 μg of total RNA were separated by gel electrophoresis on 1% agarose under denaturing conditions and subsequently blotted onto nitrocellulose (Bio-Rad, Munich, Germany). Filters were baked in a vacuum oven at 80°C for 2 h. Following pre-hybridization at 42°C in 50% formamide, 50 mM sodium phosphate, 5 × Denhardt's (1 × Denhardt: 0.2% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll), and 0.5% sodium dodecylsulphate (SDS) for 2–12 h, hybridization was carried out using cDNA probes labeled with deoxyadenosine-5'-[^{32}P]triphosphate (Megaprime DNA Labelling System; Amersham, Braunschweig, Germany) in 50% formamide, 50 mM sodium phosphate (pH 6.5), 5 × SSPE (0.18 M sodium chloride, 10 mM sodium phosphate, 1 mM ethylenediamine tetraacetic acid, pH 7.4), 5 × Denhardt's and 250 μg/ml salmon sperm DNA (Calbiochem, Bad Soden, Germany) at 50°C or 42°C for 24 h for MMP-1 and β-actin or TIMP-1, respectively.

Probes used for hybridization were a 920-bp long fragment of the original clone, K4, corresponding to the 3' terminal end of the coding sequence and the 3' untranslated part of collagenase RNA [19], a 4.05 kb *Xba* fragment of the genomic clone, TIMP-1 [20] and a 450-bp long cDNA fragment of human β-actin [21] as a housekeeping gene. Following hybridization, filters were washed twice in 2 × SSC (1 × SSC: 0.15 M sodium chloride, 0.015 M tri-sodium citrate, pH 7.0), 0.1% SDS at room temperature for 15 min and twice at hybridization temperature. Intensity of signals on X-ray film was measured by densitometry and expressed relative to the β-actin signal.

2.4. *Cytotoxicity assay*

Viability was checked 24 h after incubating the cells with $NDPO_2$ or quenchers, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [22].

2.5. *Singlet oxygen generation*

Singlet oxygen was generated by the thermal dissociation of the endoperoxide of 3,3'-(1,4-naphthylidene)dipropionate ($NDPO_2$) yielding 3,3'-(1,4-naphthylidene)dipropionate (NDP) [16]. 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 [16]. 15 min after the addition of 1 mM $NDPO_2$ the rate of $^1O_2$ generation was 3 μM/min.

3. RESULTS AND DISCUSSION

Exposure of cultured human fibroblasts to the $^1O_2$ generated from $NDPO_2$ induced an increase in collagenase mRNA 24 h after incubation (Fig. 1). The collagenase mRNA level depended on the concentration of $NDPO_2$, tested up to 3 mM. The effect on collagenase expression with 3 mM $NDPO_2$ is equivalent to that observed with 200 kJ/m^2 UV-A [3]. There was no effect on collagenase mRNA when the cells were incubated in the presence of NDP (Fig. 1, Table I). As ascertained with the MTT assay, concentrations of $NDPO_2$ up to 5 mM were non-toxic, the viability being 104 ± 4% of the controls; likewise, added NDP was non-toxic.

The increase in mRNA due to exposure of cells to $NDPO_2$-generated $^1O_2$ in D_2O-based PBS was 2-fold that in normal PBS, attributable to the larger half-life of singlet oxygen in D_2O. Sodium azide (5 mM), a potent quencher of $^1O_2$, significantly blocked the increase in the collagenase mRNA level upon exposure of cells to $NDPO_2$-generated $^1O_2$ (Table I). This apparently high concentration of azide was non-toxic, as assayed

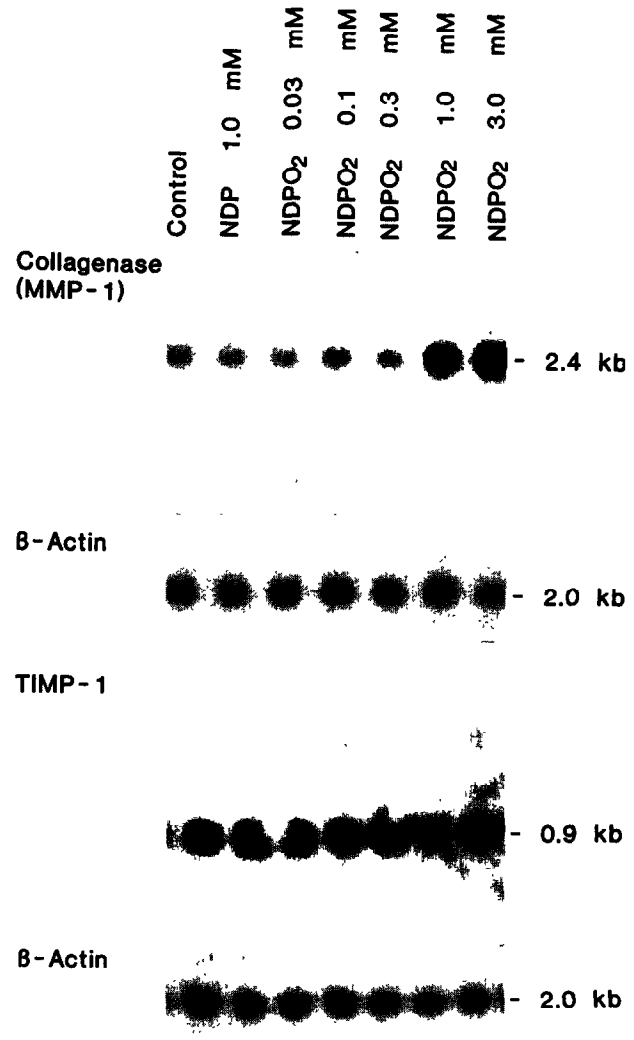

Fig. 1. Increase in collagenase (MMP-1) mRNA upon exposure of human fibroblasts to singlet oxygen generated from $NDPO_2$. For Northern blot analysis, total RNA from fibroblast monolayer cultures was isolated 24 h after incubation of cells with $NDPO_2$ (0.03–3.0 mM), separated by electrophoresis under denaturing conditions and blotted onto nitrocellulose. After hybridization with probes for human collagenase (MMP-1), TIMP-1 and β-actin, the filters were processed for autoradiography.

Table I

Collagenase, TIMP-1 and β-actin mRNA levels in fibroblast monolayers upon exposure to singlet oxygen

	Untreated	$NDPO_2$	$NDPO_2$ (D_2O)	$NDPO_2$ (Azide 5 mM)
Collagenase	1	4	8	1.2
TIMP-1	1	1	n.d.	n.d.

Data from several Northern blots were normalized to the β-actin signal (set equal to 1); $NDPO_2$ concentration was 3 mM. n.d., not determined.

with MTT (see section 2), in agreement with the observation that fibroblasts can tolerate even 100 mM azide for 2 h [6].

Taken together, these observations provide evidence that $^1O_2$ is involved in the increase in collagenase mRNA level. They support the view that UV-damage of the dermal connective tissue resulting from unbalanced synthesis of collagenase and its inhibitor (TIMP) is mediated, at least in part, by singlet oxygen. In this study, $^1O_2$ was generated in a dark reaction, leading to an induced expression of collagenase MMP-1, whereas TIMP-1 expression remained unaltered. Accordingly, $^1O_2$ released in pathological states such as inflammation may also damage the connective tissue via induced synthesis of collagenase in organs not directly accessible to light. Indeed, intracellular singlet oxygen generation was recently observed in phagocytosing neutrophils in response to particles coated with a chemical trap [23].

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