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Original Contribution

UVA RADIATION-INDUCED OXIDATIVE DAMAGE TO LIPIDS AND PROTEINS IN VITRO AND IN HUMAN SKIN FIBROBLASTS IS DEPENDENT ON IRON AND SINGLET OXYGEN

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Abstract—This study describes the damage that occurs to lipids and proteins that have been irradiated in vitro or in human skin fibroblasts with physiological doses of UVA radiation. Thiobarbituric acid-reactive species were formed from phosphatidylcholine after UVA radiation in vitro. By using iron chelators, this process was shown to involve iron. Ferric iron associated with potential physiological chelators was reduced by UVA radiation, but iron within ferritin was not. By enhancing the half life-time with deuterium oxide or by using scavengers, singlet oxygen was also shown to be involved in the UVA radiation-dependent peroxidation of phosphatidylcholine. UVA radiation-generated singlet oxygen reacted with phosphatidylcholine to form lipid hydroperoxides, and the breakdown of these hydroperoxides to thiobarbituric acid-reactive species was dependent on iron. We have shown that iron and singlet oxygen are also involved in the UVA radiation-dependent formation of thiobarbituric acid-reactive species in human skin fibroblasts, and we propose that a similar concerted effect of iron and singlet oxygen is involved in UVA radiation-dependent damage to fibroblast lipids. Sulphydryl groups of bovine serum albumin and human γ-globulin were oxidised upon UVA irradiation in vitro. The use of scavengers and deuterium oxide showed that UVA radiation-dependent sulphydryl oxidation was dependent on singlet oxygen. By adding or chelating iron, UVA radiation-dependent oxidation of sulphydryl groups of bovine serum albumin and human γ-globulin was shown to be iron-dependent. The use of catalase and hydroxyl radical scavengers demonstrated that hydrogen peroxide, but not the hydroxyl radical, was involved. The oxidation of sulphydryl groups of proteins in human skin fibroblasts that occurs as a result of UVA irradiation was also shown to involve iron, singlet oxygen, and hydrogen peroxide. We conclude that iron, singlet oxygen, and hydrogen peroxide are important redox active species involved in the deleterious effects of UVA radiation on lipids and proteins of human skin cells.

Keywords—Oxidative stress, UVA radiation, Lipid peroxidation, Sulphydryl oxidation, Human skin fibroblasts, Iron, Singlet oxygen, Free radicals

INTRODUCTION

The ultraviolet A (UVA) radiation component of sunlight (320–380 nm) has been shown to be a factor in many of the consequences of exposure of animals to sunlight including carcinogenesis and aging.[1–4] Although the mechanisms by which UVA radiation is involved in these processes are largely unknown, redox active species have been implicated.[5,6] A recent study showing that solar irradiation depletes antioxidant compounds in skin cells in situ,[7] adds to the evidence that solar radiation is a source of oxidant stress to skin.[8] Indeed, UVA irradiation of cellular constituents in vitro leads to the generation of redox active species. Hydrogen peroxide can be generated by the UVA irradiation of tryptophan,[9] and both hydrogen peroxide and superoxide can be generated from the UVA irradiation of NADH and NADPH.[10,11] Singlet oxygen can be generated as a result of absorption of UVA radiation by endogenous photosensitisers such as porphyrins.[12] The involvement of ferrous iron (Fe^{2+}) in catalysing redox reactions in vitro is well established (reviewed in ref. 13). UVA radiation has been shown to reduce ferric iron (Fe^{3+}) associated with ferritin in vitro,[14] but it is not certain that the iron was from the core since loosely bound iron that associates with preparations of ferritin[15] was not removed. Citrate-chelated ferric iron can be reduced by UVA radiation, and in the presence of hydrogen peroxide the ferrous ions can catalyse the formation of hydroxyl radicals.[16] Whether UVA radiation can reduce ferric iron bound to other potential low molecular weight intracellular chelators, besides

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citrate, or iron associated with larger cell constituents such as phosphatidylcholine, has not been investigated. Likewise, the role of hydroxyl radicals in the biological effects of UVA radiation has not been thoroughly investigated.

Ferrous ions are good catalysts for the peroxidation of membrane lipids and give rise to membrane damage in vitro.[13] Inhibition of UVA radiation-dependent peroxidation of liposomal membranes with singlet oxygen scavengers, but not iron chelators, has been demonstrated.[17] However Bose et al.[17] attempted to chelate iron in multilamellar liposomes with EDTA, a situation in which water soluble agents, such as EDTA, may be unable to access iron bound within the lipid layers. Thus, the role of iron in UVA radiation-dependent peroxidation of lipids is unknown. Ferrous ions, in conjunction with hydrogen peroxide, can also catalyse the oxidation of protein sulphydryl groups and cause the inactivation of protein function in vitro.[18–20] This process has been shown to be enhanced by UVA radiation.[21]

Exposure of cultured human skin cells to UVA radiation immediately modifies cellular components and can eventually lead to cell death.[22] The peroxidation of lipids[23,24] and the oxidation of glutathione[25] have been shown to be the immediate consequences of UVA irradiation of human skin cells and are typical of reactions of redox active species. In addition, both UVA radiation-dependent cell death and gene activation in human skin cells have been shown to involve singlet oxygen.[26,27] However, the redox active species generated by UVA irradiation of human skin cells and the immediate effects of these species are, to a large extent, unknown. The purpose of the study described here was to investigate the mechanisms of UVA radiation-dependent damage to lipids and proteins in vitro and in cultured human skin fibroblasts and to identify the biologically important redox active species that are generated intracellularly by UVA irradiation.

MATERIALS AND METHODS

In vitro analyses

UVA irradiation. For irradiations a UVASUN 3000 lamp (Mutzhaus, Munich, Germany) was used at a fluence rate of 300 Wm^{-2}. This lamp emitted wavelengths in the range of 320–410 nm. Lipids or proteins were irradiated either in phosphate buffer (25 mM, pH 7.4) or in phosphate buffered saline (PBS), as indicated, at 25°C. In some cases, buffers were made up in deuterium oxide (D_2O) instead of water. Buffers and solutions were not treated to remove adventitious metals such as iron. All biochemicals were from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Reduction of iron by UVA irradiation. Solutions of Fe^{3+} (10 μM) and chelator (100 μM) were mixed prior to being irradiated with UVA in phosphate buffer at 25°C in the presence of ferrozine (30 μM). Ferritin was either used as supplied or treated with Chelex 100 resin (Biorad, CA, USA) for 18 h. Immediately after irradiation, the absorption of a complex between Fe^{2+} and ferrozine was measured (ε_{562} = 27 900 M^{-1} cm^{-1}).

Liposomal peroxidation by UVA radiation. Phosphatidylcholine (1 mg ml^{-1}) Type XI-E 99% pure from fresh egg yolk solubilised in 1% polyoxyethylene ether W-1 was irradiated in phosphate buffer. For some experiments either multilamellar liposomes were formed by vigorously shaking phosphatidylcholine (5 mg ml^{-1}) with glass beads in 0.15 M NaCl or unilamellar liposomes were formed by sonication according to the method of Aust.[28] Liposomes at a concentration of 1 mg ml^{-1} were irradiated in phosphate buffer. Immediately after irradiation the amount of lipid peroxidation was determined from the levels of thiobarbituric acid-reactive species and results expressed as malondialdehyde (MDA) equivalents (ε_{532} = 1.56 × 10^5 M^{-1} cm^{-1}).[28] Lipid hydroperoxide levels were measured after UVA irradiation of detergent dispersed phosphotidylcholine (1 mg ml^{-1}) using the iodide method of Baker and Gebicki.[29] Results are expressed as cumene hydroperoxide equivalents (ε_{353} = 1.73 × 10^4 M^{-1} cm^{-1}).

Protein sulphydryl quantitation. The level of sulphydryl groups of bovine serum albumin, fraction V, 99% pure (5 mg ml^{-1}) and human γ-globulin, Cohn fraction II and III, 99% pure (5 mg ml^{-1}), before and after UVA radiation in PBS, was measured using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB).[30] Both proteins were dialysed against PBS to remove any possible low molecular weight sulphydryl-containing compounds such as cysteine.

Analyses in cells

Pretreatment and UVA irradiation of cells. Monolayers of human skin fibroblasts were cultured to 100% confluency as previously described[31] and used between passages 6–16. Monolayers of cells were pretreated with agents in PBS for the period of time indicated, then washed with PBS twice and UVA irradiated in PBS. Control cells were pretreated in the same manner, but received no UVA radiation.

Measurement of superoxide dismutase and catalase activities in fibroblasts after pretreatment with diethyldithiocarbamate or aminotriazol. Cells that had been treated with PBS (control) or aminotriazol (1 mM) in PBS for 1.5 h were harvested and homogenised with a Potter Elvehjem homogeniser (Belco, Felham, UK). Homogenates were centrifuged at 15 000 g for 20 min at 4°C, then aliquots of the supernatant (cytoplasm) were added to hydrogen peroxide (100 μM) in PBS, in the presence or absence of azide (20 μM), and the disappearance of hydrogen peroxide was monitored spectrophotometrically (ε_{240} = 43.6 M^{-1} cm^{-1}). Extracts from cells that had been treated with diethyldithiocarbamate (DDC, 1 mM) in PBS or PBS (control) for 1.5 h were made in the same way as for cells treated with aminotriazol. The ability of cytoplasmic extracts to inhibit the reduction of cytochrome c by a superoxide generating system (hypoxanthine (200 μM), xanthine oxidase (0.025 U ml^{-1}) and catalase (20 μg ml^{-1}) was measured in the presence and absence of the Cu-Zn superoxide dismutase inhibitor potassium cyanide (1 mM diluted to a final concentration of 10 μM for the superoxide assay). Cytochrome c reduction was measured as the change in absorbance at 550 nm ($\varepsilon_{550(\text{reduced - oxidised})}$ = 2.1 × 10^4 M^{-1} cm^{-1}).

Measurement of lipid peroxidation in fibroblasts. Immediately after irradiation, cells were harvested, counted, pelleted, and resuspended in isopropanol and hexane (2:1) to extract the lipids,[32] and butylated hydroxytoluene (0.02%) was added to prevent further oxidation. Extracts were dried under N_2, resuspended in 2.8% trichloroacetic acid, and reacted with thiobarbituric acid.[28] Cell lipid peroxidation was determined from the absorbance of the butanol extracted thiobarbituric acid-reactive species and was expressed as MDA equivalents.

Measurement of protein sulphydryl levels in fibroblasts. Immediately after irradiation, cells were harvested, counted, and pelleted, then washed three times with trichloroacetic acid (10%). The precipitated protein pellet was solublised in a sodium dodecyl sulphate buffer,[30] and the sulphydryl content was measured by addition of DTNB.[30]

RESULTS

UVA irradiation of lipids and proteins in vitro

Because ferrous ions can catalyse a number of oxidative reactions, we first investigated the ability of physiological doses of UVA radiation to reduce ferric iron associated with potential intracellular chelators. Of the low molecular weight ferric complexes that may exist in cells (ferric-citrate, -pyrophosphate, and -ADP[33]) only ferric-citrate was reduced by 250 kJ m^{-2} of UVA radiation (Table 1). Neither ferric-ADP or -pyrophosphate complexes were reduced with UVA radiation doses of up to 500 kJ m^{-2} (not shown). The amount of ferric-citrate reduced by UVA radiation was unchanged by doubling or halving the concentration of ferrozine (data not shown). This rules out the possibility that the ferric-citrate complex was reduced by a photoactive ferrozine species. Halving the concentration of ferric-citrate complex halved the amount of ferrous iron formed following irradiation with 250 kJm^{-2} of UVA (data not shown). Larger cellular constituents that have been shown to have an affinity for iron include phosphatidylcholine[34] and ferritin, the major intracellular store of nonmetabolised iron. UVA radiation could reduce ferric ions in the presence of phosphatidylcholine dispersed with detergent or in the form of unilamellar liposome, but not in the presence of phosphatidylcholine in the form of multilamellar liposomes (Table 1). Ferrous ion formation was detected after UVA irradiation of unpurified ferritin; however, following overnight treatment of ferritin with an iron chelating resin (Chelex 100), no ferrous iron was formed following UVA irradiation (Table 1). Thus, the UVA radiation-dependent reduction of iron associated with unpurified ferritin observed here and presumably by others[14] was not iron from the core of

Table 1. UVA Radiation-Dependent Reduction of Ferric Iron Bound to Potential Intracellular Chelators

Chelator	Fe^{3+} Reduced (μM)
None	undetectable
ADP (100 μM)	undetectable
Citrate (100 μM)	9.0 ± 0.5
Pyrophosphate (100 μM)	undetectable
Phosphatidylcholine (1 mg); detergent dispersed	1.5 ± 0.25
Phosphatidylcholine (1 mg); unilamellar liposomes	0.5 ± 0.25
Phosphatidylcholine (1 mg); multilamellar liposomes	undetectable
Ferritin (100 μg)	0.5 ± 0.25
Ferritin (100 μg) (Chelex 100 treated 18 h)	undetectable
EDTA (100 μM)	undetectable
Desferrioxamine (100 μM)	undetectable
PIH (100 μM)	undetectable

Incubations (1 ml) in phosphate buffer contained ferric iron (Fe^{3+}, 10 μM) or ferritin (100 μg), chelator at the concentration shown and ferrozine (30 μM), and were irradiated with 250 kJm^{-2} UVA. In no case were ferrous ions formed in the absence of irradiation. Ferric:chelator complexes were preformed before addition of buffer. The amount of ferric iron reduced was determined from the formation of the ferrous-ferrozine complex. The mean ± SD of four experiments is shown. The sensitivity of the ferrozine assay is such that levels of ferrous iron formed that were less than 0.5 μM were classed as undetectable.

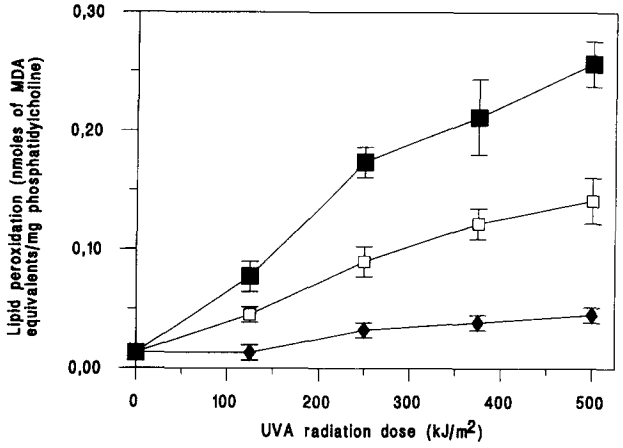

Fig. 1. UVA radiation dose-dependent peroxidation of detergent dispersed phosphatidylcholine. Incubations in phosphate buffer contained phosphatidylcholine (1 mg ml^{-1}) and additions: (□—□) none; (◆—◆) EDTA (200 μM); (■—■) deuterium oxide. Lipid peroxidation was measured as the formation of thiobarbituric acid-reactive species and is expressed as malondialdehyde (MDA) equivalents. The mean ± SD of 3 experiments is shown.

ferritin, but loosely bound iron that can associate with ferritin preparations.[15] Overnight treatment of ferritin with the iron chelating resin did not decrease the total amount of iron within the core of ferritin (data not shown). Ferric ions complexed with the nonphysiological chelators desferrioxamine, pyridoxisonicotinoylhydrazone (PIH) or ethylenediaminetetraacetic acid (EDTA) were not reduced by 250 kJm^{-2} of UVA radiation (Table 1). This result differs from that obtained in an independent study that showed that a ferric-EDTA complex could be reduced by UVA radiation at discrete wavelengths (325, 350, and 375 nm).[14] However the radiation doses used were not provided.

The next experiments were designed to determine the redox active species involved in the UVA radiation-dependent peroxidation of lipids. UVA irradiation of detergent-dispersed phosphatidylcholine led to a dose-dependent formation of lipid peroxidation end products as determined by the increase in thiobarbituric acid-reactive species (Fig. 1). UVA irradiation (250 kJ m^{-2}) of phosphatidylcholine in the form of unilamellar or multilamellar liposomes resulted in a similar level of lipid peroxidation (0.070 ± 0.007 and 0.064 ± 0.006 nmoles MDA mg^{-1} phosphatidylcholine, respectively) as for detergent dispersed phosphatidylcholine (Table 2). The iron chelator EDTA inhibited UVA radiation-dependent peroxidation of detergent dispersed phosphatidylcholine by at least 75% (Fig. 1). UVA radiation-dependent peroxidation of phosphatidylcholine in the form of unilamellar liposomes was inhibited by EDTA by only 45%, and in the case of multilamellar liposomes EDTA did not inhibit UVA radiation-dependent peroxidation at all. This implicates iron in the UVA radiation-dependent peroxidation of phosphatidylcholine and suggests that with unilamellar and multilamellar liposomes UVA radiation-dependent peroxidation occurred at sites less accessible to the aqueous phase. This interpretation is likely given that no attempt was made to minimise iron contamination during liposomal preparation. To allow access of agents to sites of UVA radiation-induced peroxidation of phosphatidylcholine all subsequent manipulations were with detergent-dispersed phosphatidylcholine. Two other iron chelators, desferrioxamine and PIH, also inhibited UVA radiation-dependent phosphatidylcholine peroxidation, whereas added iron enhanced peroxidation (Table 2). UVA radiation-dependent peroxidation of phosphatidylcholine was inhibited by 50–60% by azide and histidine (Table 2). Both these agents are good scavengers of singlet oxygen and hydroxyl radicals [rate constants (M^{-1} s^{-1}) azide; 2.2×10^9, 1.1×10^{10} and histidine; 3.2×10^7, 5×10^9 for singlet oxygen and hydroxyl radical, respectively[35,36]]. The more specific hydroxyl radical scavengers mannitol or benzoate did not effect UVA radiation-dependent phosphatidylcholine peroxidation (Table 2) [rate constants (M^{-1} s^{-1}) mannitol; 10^3, 2.7×10^9 and benzoate; unknown, 4.3×10^9 for singlet oxygen and hydroxyl radical, respectively[35,36]]. Replacement of water with deuterium oxide (which enhances singlet oxygen half-life 26-fold[37]) approximately doubled the UVA radiation dose-dependent peroxidation of phosphatidylcholine (Fig. 1). The addition of catalase (which removes hydrogen peroxide) and superoxide dismutase (which dismutates superoxide to hydrogen peroxide) did not effect UVA radiation-dependent phosphatidylcholine peroxidation (Table 2).

The UVA radiation-dependent peroxidation of detergent-dispersed phosphatidylcholine was investigated further by measuring the formation of lipid hydroperoxides, an earlier event in the lipid peroxidation chain reaction. UVA irradiation of phosphatidylcholine resulted in the formation of lipid hydroperoxides in the presence of EDTA (Table 3), which prevented iron-dependent formation of lipid peroxidation end products (Fig. 1). All subsequent measurements of lipid hydroperoxide levels were performed in the presence of EDTA. The levels of preexisting phosphatidylcholine hydroperoxides was approximately $\frac{1}{5}$ of the level induced by UVA in the presence of EDTA. Deuterium oxide enhanced the UVA-dependent formation of lipid hydroperoxides from dispersed phosphatidylcholine and the singlet oxygen/hydroxyl radical scavengers azide and histidine inhibited UVA radiation-dependent lipid hydroperoxide formation (Table 3). The specific

Table 2. Phosphatidylcholine Peroxidation and Change in the Sulphydryl Content of Bovine Serum Albumin and Human γ-Globulin Following UVA Irradiation

Addition	Thiobarbituric Acid-Reactive Species (nmoles MDA equivalents mg^{-1} phosphatidylcholine)		Sulphydryl Loss (%) 250 kJm^{-2}	
	250 kJm^{-2}	500 kJm^{-2}	Bovine Serum Albumin	Human γ-Globulin
None	0.077 ± 0.010	0.132 ± 0.11	18 ± 2	20 ± 3
Desferrioxamine (200 μM)	0.019 ± 0.006	0.037 ± 0.007	2 ± 1	1 ± 1
PIH (200 μM)	0.012 ± 0.006	0.028 ± 0.007	3 ± 1	2 ± 1
Fe^{3+} (10 μM)	0.243 ± 0.019	0.390 ± 0.024	28 ± 3	31 ± 4
Azide (10 mM)	0.032 ± 0.010	0.075 ± 0.006	3 ± 1	2 ± 1
Histidine (10 mM)	0.038 ± 0.006	0.076 ± 0.008	5 ± 2	3 ± 2
Mannitol (10 mM)	0.077 ± 0.006	0.135 ± 0.009	16 ± 3	20 ± 2
Benzoate (10 mM)	0.077 ± 0.006	0.127 ± 0.008	17 ± 2	18 ± 1
Catalase (40 μg ml^{-1})	0.071 ± 0.005	0.138 ± 0.010	16 ± 2	20 ± 2
Superoxide dismutase (80 μg ml^{-1})	0.077 ± 0.006	0.128 ± 0.010	20 ± 2	18 ± 3
Hydrogen peroxide (100 μM)	not done	not done	26 ± 3	32 ± 3

For phosphatidylcholine peroxidation mixtures in phosphate buffer contained detergent dispersed phosphotidylcholine (1 mg ml^{-1}) and additions as shown. Mixtures were irradiated with 250 kJm^{-2} or 500 kJm^{-2} of UVA. Lipid peroxidation was measured as the formation of thiobarbituric acid-reactive species as described in Materials and Methods and is shown as malondialdehyde (MDA) equivalents. The mean ± SD of three experiments with duplicates is shown with the background subtracted. Background nmoles MDA mg^{-1} phosphatidylcholine = 0.013 ± 0.006. For sulphydryl measurements mixtures in PBS contained either bovine serum albumin or human γ-globulin (5 mg ml^{-1}) and additions as shown and were irradiated with 250 kJm^{-2} UVA. Sulphydryl content of irradiated mixtures is shown relative to the sulphydryl content of unirradiated mixtures that had the same additions as the irradiated incubations. The levels of sulphydryl groups in unirradiated samples gave absorbances at 412 nm of 0.24 and 0.13 for bovine serum albumin and human γ-globulin respectively. The mean ± SD of four experiments is shown.

hydroxyl radical scavengers benzoate and mannitol had no effect on lipid hydroperoxide formation (Table 3). The UVA radiation-dependent accumulation of lipid hydroperoxides from phosphatidylcholine was inhibited by scavengers to a degree that reflected their reactivity with singlet oxygen, that is, azide > histidine > mannitol.

Table 3. Lipid Hydroperoxide Accumulation Following UVA Irradiation of Phosphatidylcholine

Addition	Lipid Hydroperoxide Accumulation (nmoles cumene hydroperoxide equivalent mg^{-1} phosphatidylcholine)
None	0.40 ± 0.12
EDTA (200 μM)	1.62 ± 0.17
EDTA (200 μM) + deuterium oxide	2.65 ± 0.11
EDTA (200 μM) + azide (10 mM)	0.23 ± 0.05
EDTA (200 μM) + histidine (10 mM)	0.57 ± 0.12
EDTA (200 μM) + mannitol (10 mM)	1.62 ± 0.17
EDTA (200 μM) + benzoate (10 mM)	1.68 ± 0.12

Mixtures in phosphate buffer contained detergent-dispersed phosphatidylcholine (1 mg ml^{-1}) and additions as shown, and were irradiated with 250 kJm^{-2} UVA. Lipid hydroperoxide levels were measured by the iodide method as described in Materials and Methods. The mean ± SD of three experiments is shown with the level of preexisting phosphatidylcholine hydroperoxides subtracted. Preexisting cumene hydroperoxide equivalents mg^{-1} phosphatidylcholine = 0.28 ± 0.06.

We also investigated the effects of UVA irradiation on sulphydryl groups of isolated proteins. Irradiation of bovine serum albumin with UVA resulted in a dose-dependent loss of the sulphydryl content (Fig. 2). A similar radiation dose-dependent loss of sulphydryls was seen with human γ-globulin (not shown). The iron chelators EDTA (Fig. 2), desferrioxamine and PIH (Table 2) inhibited the UVA radiation-dependent loss of sulphydryl groups of bovine serum albumin and human γ-globulin, as did the singlet oxygen/hydroxyl radical scavengers azide and histidine (Table 2). Irradiation of bovine serum albumin or human γ-globulin in the presence of deuterium oxide (Fig. 2), iron, or hydrogen peroxide (Table 2) increased the level of protein sulphydryl loss. The hydroxyl radical scavengers mannitol or benzoate had no effect on the UVA radiation-dependent loss of sulphydryl groups, nor did irradiation in the presence of superoxide dismutase or catalase (Table 2).

UVA irradiation of human skin fibroblasts

UVA irradiation of human skin keratinocytes and fibroblasts has been shown to lead to the peroxidation of membrane lipids[23,24] though the mechanism of peroxidation has not been investigated. The results of this

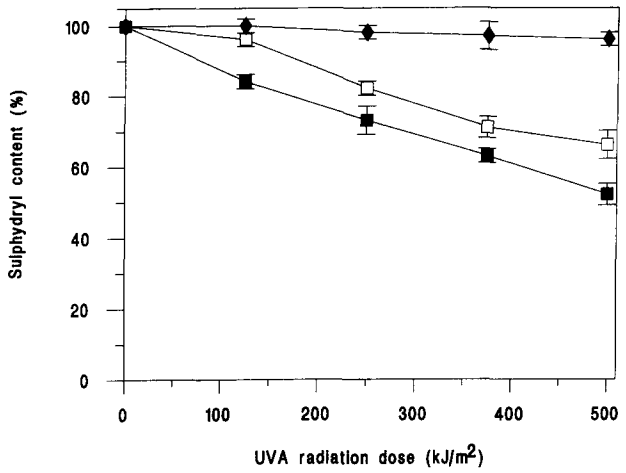

Fig. 2. UVA radiation dose-dependent change in the sulphydryl content of bovine serum albumin. Incubations in phosphate buffer contained bovine serum albumin (5 mg ml^{-1}) and additions: (□—□) none; (◆—◆) EDTA (100 μM); (■—■) deuterium oxide. The mean ± SD of 3 experiments is shown.

study, which implicate singlet oxygen and iron in the UVA radiation-dependent peroxidation of phosphatidylcholine, encouraged us to investigate the mechanism of lipid peroxidation in human skin fibroblasts induced by UVA irradiation. Irradiation of fibroblasts led to a UVA dose-dependent formation of thiobarbituric acid-reactive species (Fig. 3). Because we measured levels of thiobarbituric acid-reactive species in lipid extracts of cells made after UVA irradiation, we have assumed that we have measured peroxidation of cell lipids and not oxidation products of other cell molecules that have been shown to interfere with the assay used.[38] All chemical treatments of cells involved a pretreatment followed by thorough washing to ensure that, as much as possible, the effects of the agents were intracellular. UVA radiation-dependent lipid peroxidation was inhibited by 70% by high concentrations of desferrioxamine (Table 4), an iron chelator that does not readily enter cells, but can decrease intracellular levels of iron[39] and can protect against dye-sensitised phototoxicity.[40] Ferrioxamine (iron loaded desferrioxamine) had no effect on UVA radiation-dependent lipid peroxidation (data not shown). PIH, an iron chelator that can more readily pass through the membrane and can bind intracellular iron,[41] had no effect on UVA radiation-dependent cell lipid peroxidation at a maximal nontoxic concentration (Table 4). Deuterium oxide enhanced the level of UVA radiation-dependent lipid peroxidation by 35% and histidine inhibited lipid peroxidation by 43%. Azide was not used for cell experiments because of its ability to inhibit catalase. The hydroxyl radical scavengers mannitol and benzoate had no effect on UVA radiation-dependent cell lipid peroxidation (Table 4). Pretreatment of cells with aminotriazol inhibited catalase levels by 86% (Table 5) and inhibited UVA radiation-dependent lipid perox-

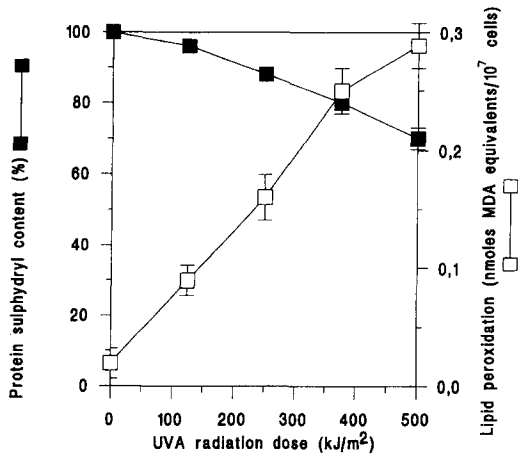

Fig. 3. The UVA radiation dose-dependent peroxidation of lipids and change in protein sulphydryl content of human skin fibroblasts. Lipid peroxidation (□—□) was measured as the formation of thiobarbituric acid reactive species in lipid extracts of cells ($1-5 \times 10^7$) after UVA irradiation, and is expressed as malondialdehyde (MDA) equivalents. Protein sulphydryl content (■—■) was measured in protein extracts of cells ($1-5 \times 10^6$) after UVA irradiation. The mean ± SD of three experiments for lipid peroxidation and five experiments for sulphydryl content are shown.

Table 4. Peroxidation of Lipids and Changes in the Protein Sulphydryl Content of Human Skin Fibroblasts Following UVA Irradiation

Pretreatment	Thiobarbituric Acid-Reactive Species (nmoles MDA equivalents 10^{-7} cells)	Cell Protein Sulphydryl Loss (%)
None	0.15 ± 0.01	12 ± 2
Desferrioxamine (5 mM)	0.05 ± 0.03	13 ± 2
PIH (1 mM)	0.15 ± 0.02	3 ± 2
Deuterium oxide	0.20 ± 0.03	18 ± 2
Histidine (10 mM)	0.08 ± 0.01	5 ± 1
Mannitol (10 mM)	0.13 ± 0.01	11 ± 1
Benzoate (10 mM)	0.14 ± 0.01	12 ± 1
Aminotriazol	0.10 ± 0.01	21 ± 4
Diethyldithiocarbamate	0.15 ± 0.02	12 ± 2

Prior to irradiation, cells were pretreated with additions as shown for 1.5 h, washed thoroughly and then irradiated with 250 kJm^{-2} UVA. Peroxidation was measured as the amount of thiobarbituric acid-reactive species in lipid extracts of cells ($1-5 \times 10^7$) made after UVA irradiation. Levels are shown as MDA equivalents. The mean ± SD of four experiments is shown with the background level of peroxidation subtracted. Background nmoles MDA equivalents 10^{-7} cells = 0.02 ± 0.01. For sulphydryl measurements protein extracts of cells ($1-5 \times 10^6$) were made immediately after irradiation. Protein sulphydryl content of irradiated cells are shown relative to the protein sulphydryl content of unirradiated cells that had received the same additions. The mean ± SD of three experiments is shown.

Table 5. Levels of (A) Catalase and (B) Superoxide Dismutase in Human Skin Fibroblasts After Treatment With PBS (control), Diethyldithiocarbamate (DDC), or Aminotriazol

	Catalase Activity (nmol H_2O_2 consumed min^{-1} mg^{-1} protein)
A. Control extract	44 ± 2
Control extract + axide (20 μM)	0 ± 0.5
Aminotriazol extract	6 ± 2
Aminotriazol extract + azide (20 μM)	0 ± 0.5
	Cytochrome c Reduced (nmol min^{-1} mg^{-1} protein)
B. $O_2^{\bullet-}$ generating system	15.2 ± 1
$O_2^{\bullet-}$ generating system + control extract	8.1 ± 0.5
$O_2^{\bullet-}$ generating system + control extract + cyanide (10 μM)	13.1 ± 1.0
$O_2^{\bullet-}$ generating system + DDC treated extract	12.8 ± 0.4
$O_2^{\bullet-}$ generating system + DDC treated extract + cyanide (10 μM)	13.4 ± 0.5

Enzyme activity was determined in cell extracts as described in the Materials and Methods, and the values shown are the mean ± range of two experiments. Catalase activity was determined as the difference between the activity of the control extract minus activity of the control extract with azide (44 nmol min^{-1} mg^{-1} protein) and the activity of the aminothiazol extract minus the activity of the aminotriazol extract with azide (6 nmol min^{-1} mg^{-1} protein). Total superoxide dismutase activity was calculated from the difference between the rate of cytochrome c reduction of the system and the system plus extract (7.1 and 2.4 nmol cytochrome c reduced min^{-1} mg^{-1} protein for control and DDC extracts, respectively). The Cu-Zn superoxide dismutase activity of the extracts was calculated from the difference between the rates of cytochrome c reduction of the extracts and the extracts plus cyanide (5.0 and 0.6 nmol cytochrome c reduced min^{-1} mg^{-1} protein for control and DDC extracts, respectively).

idation by 30% (Table 4). Diethyldithiocarbamate pretreatment of cells inhibited Cu-Zn superoxide dismutase levels by 88% and total superoxide dismutase levels by 66% (Table 5), but did not effect UVA radiation-dependent lipid peroxidation (Table 4).

Because it has been shown that UVA irradiation of human skin fibroblasts leads to the oxidation of intracellular glutathione,[25] we were interested to determine whether sulphydryl groups of intracellular proteins may also be effected. UVA radiation decreased the protein sulphydryl content of human skin fibroblasts in a dose-dependent manner (Fig. 3). Oxidation of protein sulphydryls was prevented by the intracellular iron chelator PIH, but not desferrioxamine (Table 4). Deuterium oxide enhanced, whereas histidine decreased, the UVA radiation-dependent oxidation of protein sulphydryl groups. The hydroxyl radical scavengers mannitol and benzoate had no effect on the UVA radiation-dependent loss of protein sulphydryl groups. Cells with low catalase activity (aminotriazol treated, Table 5) lost a greater proportion of sulphydryls after UVA radiation compared to normal cells (Table 4). Decreasing the cellular content of Cu-Zn superoxide dismutase (with diethyldithiocarbamate, Table 5) had no effect on the UVA radiation-dependent oxidation of sulphydryl groups (Table 4).

DISCUSSION

Here we identify ferrous iron, singlet oxygen, and hydrogen peroxide as biologically important redox active species generated by UVA irradiation of human skin fibroblasts. These redox active species were shown to mediate a UVA radiation-dependent loss of protein sulphydryl groups in vitro and in human skin fibroblasts, and singlet oxygen and iron were shown to mediate UVA radiation-dependent peroxidation of phosphatidylcholine and fibroblast lipids. The amount of UVA radiation used in these studies (250 kJm^{-2}) was equivalent to the amount of UVA radiation that would reach the surface of the skin during 70 min of sunlight exposure at noon, during a cloudless summer day, at a northern latitude of 35°.[42] The amount of UVA radiation penetrating to the basal layer of the epidermis would be approximately 30% of this dose.[43] A UVA radiation dose of 250 kJm^{-2} kills 15 ± 5% of cultured human skin fibroblasts (FEK4 as used in this study)[26] and releases 4 ± 0.5% of the total lactate dehydrogenase content of these fibroblasts in culture.[44]

In this study we have shown that physiological doses of UVA radiation reduce ferric iron associated with phosphatidylcholine or bound to the potential low molecular weight intracellular chelator citrate. Van der Zee et al.[14] proposed that the UV/near visible radiation-dependent reduction of ferric ions associated with citrate occurred via the light-induced transfer of electrons from citrate to iron. The ability of UVA radiation to reduce iron associated with phosphatidylcholine in the form of unilamellar liposomes or dispersed with detergent, but not phosphatidylcholine in the form of multilamellar liposomes, suggests that if a species capable of electron transfer is generated upon UVA irradiation of liposomes, then the electron cannot be transferred to the aqueous phase. Any adventitious iron within the multilamellar liposomal bilayers that may have been reduced by UVA radiation would not be detected in our system because the molecule that detects ferrous iron was located in the aqueous phase. Because UVA radiation can reduce ferric iron associated with cellular molecules, it should be able to initiate biological redox reactions in which ferrous ions have been shown to have a catalytic role. Such reac-

tions include lipid peroxidation,[13] sulphydryl oxidation,[18-20] and the generation of subsequent redox active species such as the hydroxyl radical.[45] Indeed, this study clearly shows that the UVA radiation-dependent peroxidation of detergent dispersed phosphatidylcholine is dependent on iron in agreement with the general hypothesis that iron is an essential component in redox-driven lipid peroxidation systems.[13] The role of iron in lipid peroxidation has been shown to be in the breakdown of lipid hydroperoxides (LOOH) according to reaction [1]:

$$LOOH + Fe^{2+} \rightleftharpoons LO^{\cdot} + OH^{-} + Fe^{3+} \quad (1)$$

The buffers and solutions used in this study would have contained sufficient adventitious iron to catalyse such reactions.[46] EDTA has been shown to inhibit lipid peroxidation of multilamellar liposomes[34,47] in systems where the reduction of ferric iron occurs in the aqueous phase. The inability of EDTA to inhibit UVA-dependent lipid peroxidation of multilamellar liposomes shown here, and by others,[17] suggests that the UVA radiation-dependent induction of peroxidation of phosphatidylcholine liposomes occurs at sites within the lipid. Thus, an important feature of the biological effects of UVA radiation may be the ability of UVA radiation to interact with iron within lipid bilayers.

In agreement with another study,[17] our experiments here are consistent with the involvement of singlet oxygen in the UVA radiation-dependent formation of thiobarbituric acid-reactive species from phosphatidylcholine. Further investigation revealed that singlet oxygen ($^1O_2$) was involved at the level of the formation of phosphatidylcholine hydroperoxides. This is probably via reaction [2] as described by Foote[48] and others[49]:

$$^1O_2 + LH \rightleftharpoons LOOH \quad (2)$$

Thus, we propose that UVA radiation can break down phosphatidylcholine to thiobarbituric acid-reactive species via the concerted action of singlet oxygen and ferrous iron. The involvement of iron and singlet oxygen in the UVA radiation-dependent peroxidation of fibroblast lipids is consistent with the possibility that a mechanism, similar to that seen with phosphatidylcholine, is involved in fibroblasts. We could find no evidence for the involvement of superoxide or the hydroxyl radical in the UVA radiation-dependent peroxidation of phosphatidylcholine or cell lipids. The lack of involvement of hydroxyl radicals in UVA radiation-dependent lipid peroxidation that we show is in agreement with others who have concluded that hydroxyl radicals are not able to induce lipid peroxidation.[13,34,50] The partial inhibition of UVA radiation-dependent peroxidation of lipid in cells with low levels of catalase activity (aminotriazol treated) suggests that hydrogen peroxide may compete with UVA radiation-generated lipid hydroperoxide for the ferrous iron via reaction [3] as described by Girotti[51]:

$$Fe^{2+} + H_2O_2 \rightleftharpoons OH^{\cdot} + OH^{-} + Fe^{3+} \quad (3)$$

UVA irradiation of human skin fibroblasts at physiological doses has been shown to lead to a loss of intracellular glutathione,[25] and we now show that similar doses of UVA radiation can lead to a loss of protein sulphydryl groups both in these cells and in vitro. The UVA radiation-induced protein sulphydryl loss was shown to involve iron and hydrogen peroxide, but not hydroxyl radicals. This is in agreement with other studies that have shown that the redox-dependent loss of sulphydryl groups requires iron and hydrogen peroxide, and that the hydroxyl radical is not involved.[18-20] The mechanism by which hydrogen peroxide enhances the oxidation of protein sulphydryl groups is unknown. However, Vile and Winterbourn[20] have proposed that hydrogen peroxide may mediate the oxidation of an iron-sulphydryl complex. Singlet oxygen readily reacts with protein sulphydryl groups,[52] and we show that, in addition to the hydrogen peroxide/iron dependent mechanism, another pathway of UVA radiation-dependent loss of protein sulphydryl groups occurs via singlet oxygen both in vitro and in human fibroblasts. Loss of protein sulphydryl groups can lead to inactivation of protein function such as Ca^{2+} transport by Ca^{2+}-ATPase.[18-20] Inactivation of sulphydryl containing proteins may be an important consequence of UVA irradiation. Indeed, UVA irradiation of skin cells in situ leads to inactivation of critical antioxidant enzymes,[7] although whether this occurred via the oxidation of sulphydryl groups was not determined.

This study has identified damage to lipids and sulphydryl containing proteins as consequences of physiological doses of UVA irradiation on human skin cells. We treated cells with agents that decreased or increased levels of redox active species in order to implicate such species in UVA radiation-dependent damage to lipids and proteins. The effects of using redox modifying agents can be limited by the accessibility of the agents to intracellular sites of UVA radiation-generated damage. This is apparent from two observations. First, the effects of the agents in vitro were in most cases greater than in cells; and second, that although both desferrioxamine and PIH inhibited UVA radiation-dependent lipid peroxidation and sulphydryl oxi-

dation in vitro, their ability to inhibit these processes in fibroblasts differed. Thus, in the case of iron-catalysed UVA radiation-dependent oxidative reactions in human skin cells described in this study, the sites of intracellular iron localisation appears to be important.

The results using redox modifying agents that we obtained in this study allowed us to identify the biologically important redox active species generated by UVA irradiation of human skin fibroblasts. Singlet oxygen was identified by the opposing effects of deuterium oxide and histidine on UVA radiation-dependent lipid peroxidation and sulphydryl loss. Endogenous photosensitisers are probable sources of singlet oxygen during UVA radiation of the fibroblasts.[12] The effect of at least one of the iron chelators on both processes identified ferrous iron as being an important species generated by UVA radiation of cells. The effect of decreasing intracellular catalase levels on UVA radiation-dependent sulphydryl loss and lipid peroxidation is good evidence that hydrogen peroxide was generated intracellularly during UVA irradiation. Reaction of ferrous iron and hydrogen peroxide via reaction [3] raises the possibility that hydroxyl radicals will be generated intracellularly during UVA irradiation. However, it is clear from this study and other studies[26,27] that hydroxyl radicals are not involved in any of the cellular effects of UVA irradiation so far investigated. The lack of effect of catalase on UVA radiation-dependent loss of bovine serum albumin and human γ-globulin sulphydryl groups and phosphatidylcholine peroxidation indicates that hydrogen peroxide was probably not generated upon UVA irradiation of these in vitro systems. Our results have shown that UVA radiation could not reduce and release iron from the core of ferritin in vitro. However, the possibility that the release of iron from ferritin occurs as a result of redox active species generated by UVA radiation of cells is not ruled out by this study.

Previous studies from this laboratory have implicated redox active species in some of the biological effects of UVA irradiation of human skin cells. In particular, singlet oxygen has been shown to be involved in the UVA radiation-dependent killing of human skin fibroblasts[26] and the activation of haem oxygenase-1 messenger RNA transcription.[27] These studies and the study presented herein suggest that in addition to singlet oxygen, iron and hydrogen peroxide should be considered as mediators in the consequences of UVA irradiation on human skin cells.

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