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Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts

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ABSTRACT **Oxidative stress of human skin fibroblasts by treatment with ultraviolet A (UVA) radiation has been shown to lead to an increase in levels of the heme catabolizing enzyme heme oxygenase 1 [heme, hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3] and the iron storage protein ferritin. Here we show that human skin fibroblasts, preirradiated with UVA, sustain less membrane damage during a subsequent exposure to UVA radiation than cells that had not been preirradiated. Pretreating cells with heme oxygenase 1 antisense oligonucleotide inhibited the irradiation-dependent induction of both the heme oxygenase 1 enzyme and ferritin and abolished the protective effect of preirradiation. Inhibition of the UVA preirradiation-dependent increase in ferritin, but not heme oxygenase, with desferrioxamine also abolished the protection. This identifies heme oxygenase 1 as a crucial enzymatic intermediate in an oxidant stress-inducible antioxidant defense mechanism, involving ferritin, in human skin fibroblasts.**

Expression of the heme oxygenase 1 gene is enhanced by oxidative stress (1–6) including UVA radiation (320–380 nm) (7–9), a major component of sunlight. We have recently demonstrated that induction of the heme oxygenase enzyme [heme, hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3] by UVA irradiation of cultured human skin fibroblasts leads to an increase in ferritin (10). Ferritin constitutes the major storage site for nonmetabolized intracellular iron and therefore plays a critical role in regulating the availability of iron to catalyze such harmful reactions as the peroxidation of lipids and the Fenton reaction producing hydroxyl radicals. Recent studies have implicated intracellular ferritin in the protection of rat kidney (11) and cultured aortic endothelial cells (12) from oxidant-induced damage. This study was undertaken to determine whether a heme oxygenase-dependent increase in ferritin levels leads to an adaptive response in human skin fibroblasts.

MATERIALS AND METHODS

Cell Culture and Irradiation. Monolayers of normal human skin fibroblasts (FEK_4) were cultured to 100% confluency as described (13). For preirradiation, medium was removed and reserved and cells were washed thoroughly with phosphate-buffered saline (PBS) and covered in PBS containing Ca^{2+} and Mg^{2+} (0.01% each). Monolayers of cells were then sham preirradiated (kept under subdued white light) or preirradiated with UVA (250 $kJ \cdot m^{-2}$) at 25°C. All irradiations were with broad spectrum UVA using a Uvasun 3000 lamp (Mutzhas, Munich) at a dose rate of 2.6×10^2 $W \cdot m^{-2}$. The original medium was replaced after preirradiation. For experiments in which cells were exposed to a second dose of UVA radiation, the second irradiation was in PBS without added Ca^{2+} or Mg^{2+}.

Lactate Dehydrogenase Measurements. After the second dose of UVA radiation, 5×10^5 cells were left in the irradiation buffer (PBS) for 2 h at 37°C, after which aliquots of PBS were removed and spun at $5000 \times g$. Lactate dehydrogenase content of the supernatants was determined from the consumption of NADH as described (14). In some cases, lactate dehydrogenase leakage was expressed as a percentage of the total lactate dehydrogenase, determined by treating monolayers of cells with 0.025% Nonidet P-40 detergent in PBS, leaving for 30 min at 37°C, and then assaying an aliquot of the supernatant as described above. A unit of lactate dehydrogenase activity was defined as 1 μmol of NADH consumed per min.

Heme Oxygenase 1 Sense and Antisense Oligonucleotide Treatment. For all experiments involving treatment with oligonucleotides, cells were cultured in medium in which the fetal calf serum was substituted with 10% Nu-Serum (Paesel and Lorei, Frankfurt) as it lacked nuclease activity (data not shown). *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mannheim) was used as a vehicle for transfecting cells with the oligonucleotides. The proportions used were 2 μg of oligonucleotide/5 μg of DOTAP per ml of medium and the protocol used was as described by the manufacturer. The oligomers used were 15 nucleotides long and were directed against the region in the cDNA flanking 6 nucleotides on either side of the translation initiation codon (15) in both antisense and sense orientation. Monolayers of cells at ≈60% confluency were treated with oligonucleotide and DOTAP. Cells were incubated with the oligonucleotides for 24 h, at which stage the cells had reached 100% confluency and could be irradiated.

Heme Oxygenase 1 Protein Detection. Cells were harvested 14 h after preirradiation with 250 $kJ \cdot m^{-2}$ [when heme oxygenase enzyme activity has been shown to be maximal (10)] and protein levels were visualized by immunoblotting with polyclonal rabbit antiserum directed against rat heme oxygenase 1 protein, kindly provided by Brian Murphy (SRI International, Menlo Park, CA). Briefly, 75 μg of total cytoplasmic protein was separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:2500 dilution of the anti-heme oxygenase 1 antibody, washed, and subsequently probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1:7500. Chemiluminescent detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions. Quantitation of the levels of protein was performed by densitometric scanning of x-ray film exposed to the chemiluminescence. It should be noted that the intensity of the chemiluminescent signal is not linearly related to the amount of protein such that the quantitation would underestimate higher amounts of protein.

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Abbreviation: DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate.

Desferrioxamine Treatment. Immediately after UVA preirradiation, the irradiation buffer was removed and cells were thoroughly washed with PBS. Desferrioxamine treatment (500 μM in PBS) was for 1.5 h and then cells were washed thoroughly with PBS and the reserved medium was replaced for 22.5 h before subsequent treatments and assays.

Ferritin Measurement. At 24 h after preirradiation, 1×10^6 cells were harvested with a rubber policeman and homogenized with a Potter–Elvehjem homogenizer (Belco, Felham, U.K.), and the supernatant, after centrifugation at $5000 \times g$, was assayed for ferritin with an enzyme-linked immunosorbent assay kit (Boehringer Mannheim) according to the protocol supplied by the manufacturer. Protein content of the supernatants was measured according to the method of Bradford (16).

Lipid Peroxidation. At 2 h after the second dose of UVA radiation, 1×10^7 cells were harvested with a rubber policeman, counted, and pelleted at $1000 \times g$. Lipid was extracted from the cells with 20 vol of hexane/isopropanol (3:2) (17), containing 0.02% butylated hydroxytoluene to prevent lipid peroxidation during subsequent handling. Aliquots of the lipid extract were dried under nitrogen, resuspended in trichloroacetic acid (2.8%), and reacted with thiobarbituric acid (1%). The absorbance of the butanol-extracted thiobarbituric acid-reactive species at 532 nm was taken as an estimate of the peroxidation of membrane lipids (18).

RESULTS AND DISCUSSION

We have previously shown that ferritin levels increase 2-fold 22–46 h after treatment of human skin fibroblasts with UVA radiation (10), a finding that led us to predict that these cells would be protected from subsequent oxidative treatments. Thus, we examined fibroblasts that had either been preirradiated with UVA at 250 kJ·m^{-2} or sham preirradiated 24 h prior to being exposed to a second dose of UVA radiation. To estimate cell damage arising from the immediate effects of oxidative stress promoted by the second dose of UVA irradiation, we measured the leakage of a cytoplasmic protein, lactate dehydrogenase, into the irradiation buffer. This assay reflects cell membrane integrity, and the UVA radiation-dependent loss of membrane integrity is thought to be a consequence of UVA radiation-dependent peroxidation of membrane lipids (19). Cells that had been preirradiated 24 h previously showed a 2-fold increase in ferritin (Table 1; ref. 10) and leaked less lactate dehydrogenase with increasing UVA radiation than sham preirradiated cells as shown by a typical experiment illustrated in Fig. 1. The mean ± SD of the gradients of the dose–response curves of six experiments of the type shown in Fig. 1 was $0.044 \pm 0.014 \times 10^{-3}$ unit of lactate dehydrogenase·kJ^{-1}·m^2 for preirradiated cells and $0.096 \pm 0.010 \times 10^{-3}$ unit of lactate dehydrogenase·kJ^{-1}·m^2 for sham preirradiated cells. The total amount of lactate dehydrogenase in fibroblasts 24 h after preirradiation or sham preirradiation was the same (0.82 ± 0.06 unit).

Table 1. Ferritin content of human skin fibroblasts treated with heme oxygenase 1 antisense or sense oligonucleotide 24 h after UVA preirradiation (250 kJ·m^{-2})

Treatment	Ferritin content, ng per mg of protein
Sham preirradiated	81 ± 10
Preirradiated	194 ± 15*
Preirradiated + heme oxygenase 1 antisense oligonucleotide	135 ± 10*†
Preirradiated + heme oxygenase 1 sense oligonucleotide	194 ± 12*

Values are means ± SD of six observations. *, Significantly different from sham preirradiated ($P < 0.005$); †, significantly different from preirradiated plus heme oxygenase 1 sense oligonucleotide ($P < 0.005$).

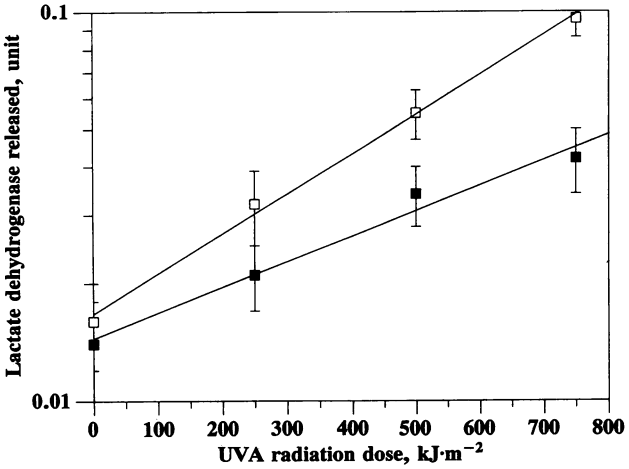

FIG. 1. Lactate dehydrogenase leakage after UVA irradiation (0–750 kJ·m^{-2}) of human skin fibroblasts 24 h after UVA preirradiation (250 kJ·m^{-2}) (■) or sham preirradiation (□). Each point represents the mean ± SD of three observations from a typical experiment.

Since we have previously shown that the UVA radiation-dependent increase in ferritin levels in human skin fibroblasts was dependent on an increase in heme oxygenase enzyme activity (10), we were interested in determining the role of heme oxygenase 1 induction on the protection observed 24 h after preirradiation. To do this, we treated human skin

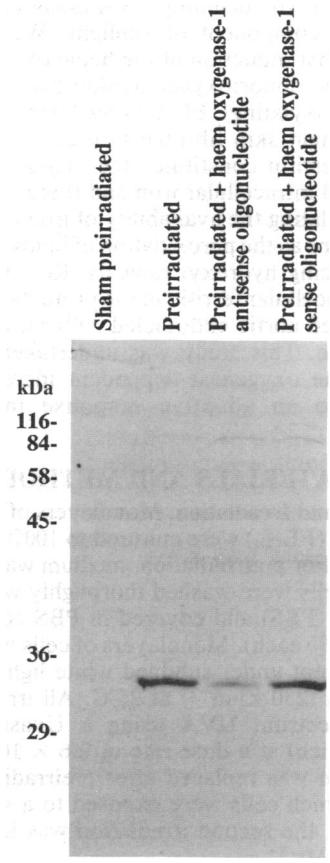

FIG. 2. Heme oxygenase 1 protein expression in cells treated with heme oxygenase 1 antisense or sense oligonucleotide 14 h after UVA preirradiation (250 kJ·m^{-2}). Heme oxygenase 1 protein is the major band appearing at 32 kDa.

fibroblasts with an antisense oligonucleotide targeted to the 5′ end of the heme oxygenase 1 mRNA. Fibroblasts treated with heme oxygenase 1 antisense oligonucleotide prior to preirradiation with UVA (250 kJ·m^{-2}) showed less of an increase in heme oxygenase 1 protein than cells containing either heme oxygenase 1 sense oligonucleotide or no oligonucleotide at all (Fig. 2). Densitometric quantitation revealed that the level of heme oxygenase 1 protein induced in antisense oligonucleotide-treated cells by UVA irradiation was 25% of the level of heme oxygenase 1 protein induced by UVA irradiation of sense oligonucleotide-treated cells. Fibroblasts treated with heme oxygenase 1 antisense oligonucleotide lost most of the protection against lactate dehydrogenase leakage afforded by preirradiation, whereas cells treated with heme oxygenase 1 sense oligonucleotide and preirradiated maintained the same degree of protection as preirradiated cells not treated with oligonucleotide (Table 2).

Since release of iron via the heme oxygenase-mediated catabolism of heme appears to be a trigger for ferritin induction (10, 20), we have further examined the role of ferritin in the protection against lactate dehydrogenase leakage by addition of desferrioxamine to cells after preirradiation. Cells that had been treated with desferrioxamine showed a complete loss of preirradiation-dependent protection compared to cells not treated with desferrioxamine (Table 2). This effect of desferrioxamine was due to its iron-chelating ability, since ferrioxamine (iron-saturated desferrioxamine) did not abolish the preirradiation-induced protection toward subsequent irradiation (data not shown). If ferritin were responsible for the protection that resulted from UVA preirradiation, then the heme oxygenase 1 antisense oligonucleotide treatment that diminished preirradiation protection should also diminish the increase in ferritin seen after preirradiation. Indeed, cells treated with heme oxygenase 1 antisense oligonucleotide did show a diminished increase in ferritin after preirradiation, whereas heme oxygenase 1 sense oligonucleotide-treated cells showed an increase in ferritin after preirradiation similar to cells not treated with oligonucleotide (Table 1). Cells treated with heme oxygenase 1 antisense oligonucleotide did not completely lose the protection afforded by preirradiation (Table 2), because this treatment did not completely prevent the preirradiation-dependent induction of heme oxygenase 1 protein (Fig. 2) and thus did not entirely abolish the subsequent induction of ferritin (Table 1).

Table 2. Effect of heme oxygenase 1 antisense and sense oligonucleotides and desferrioxamine (500 μM) on amount of lactate dehydrogenase (LDH) leakage from human skin fibroblasts after UVA irradiation (750 kJ·m^{-2}) 24 h after UVA preirradiation (250 kJ·m^{-2}) or sham preirradiation

	% LDH released by UVA irradiation (750 kJ·m^{-2})	
Addition	Sham preirradiated	Preirradiated
No addition	9.1 ± 1.1	3.8 ± 1.0*
Heme oxygenase 1 antisense oligonucleotide	9.2 ± 1.0	7.4 ± 0.9*†
Heme oxygenase 1 sense oligonucleotide	9.2 ± 0.9	4.0 ± 1.0*
Desferrioxamine	9.3 ± 0.7	9.1 ± 0.8

Each value is the mean ± SD of nine observations. *, Significantly different from sham preirradiated ($P < 0.05$); †, significantly different from preirradiated plus heme oxygenase 1 sense oligonucleotide ($P < 0.005$).

Table 3. Effect of UVA preirradiation (250 kJ·m^{-2}) and desferrioxamine treatment (500 μM) on lipid peroxidation of fibroblasts after UVA irradiation with 750 kJ·m^{-2}

Treatment	A_{532} per 10^9 cells
Sham preirradiated	1.43 ± 0.30
Preirradiated	0.47 ± 0.22*
Preirradiated + desferrioxamine	1.46 ± 0.46
Desferrioxamine	1.39 ± 0.38

Values shown are means ± SD of five observations. *, Significantly different from sham preirradiated ($P < 0.005$).

While the leakage of lactate dehydrogenase gives an indirect measure of the amount of UVA radiation-induced damage to fibroblasts, we were interested in determining whether the protection toward lactate dehydrogenase leakage after UVA preirradiation was reflected in a more direct measure of oxidant damage to the cells. We therefore measured the amount of membrane lipid peroxidation after UVA irradiation, 24 h after preirradiation or sham preirradiation. The formation of thiobarbituric acid-reactive species from a lipid extract of the cells was taken as an estimate of membrane lipid peroxidation. Preirradiation of cells decreased the level of lipid peroxidation after the second dose of UVA, 24 h later, by ≈3-fold (Table 3). Total levels of peroxidized lipid in preirradiated and sham preirradiated cells were the same immediately before the second irradiation (A_{532} per 10^9 cells = 0.12 ± 0.08). Desferrioxamine prevented the protective effect of preirradiation toward membrane lipids (Table 3). Since desferrioxamine inhibits the UVA radiation-dependent increase in ferritin, but not heme oxygenase (10), it is likely that ferritin is ultimately responsible for limiting UVA radiation-dependent oxidative damage to fibroblasts. Desferrioxamine treatment 24 h before irradiation did not affect the UVA radiation-dependent peroxidation of fibroblast membrane lipids (Table 3) or leakage of lactate dehydrogenase (Table 2). This may be because the UVA radiation-dependent lipid peroxidation and lactate dehydrogenase leakage we measured are catalyzed by iron present in the membranes that is unaffected by the desferrioxamine treatment utilized in this study (cf. ref. 21). The large quantity of oligonucleotide and DOTAP required to treat the number of cells necessary to measure lipid peroxidation ruled out the possibility of examining the effect of heme oxygenase 1 antisense/sense on the UVA radiation-induced protection toward lipid peroxidation.

In summary, we have observed an adaptive response to UVA-dependent oxidative stress in human skin cells. Our results clearly implicate heme oxygenase 1 as the initial inducible mediator in this adaptive process and implicate ferritin as an important oxidant stress-inducible antioxidant in these cells.

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