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# Ultraviolet A radiation induces immediate release of iron in human primary skin fibroblasts: The role of ferritin

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**ABSTRACT** **In mammalian cells, the level of the iron-storage protein ferritin (Ft) is tightly controlled by the iron-regulatory protein-1 (IRP-1) at the posttranscriptional level. This regulation prevents iron acting as a catalyst in reactions between reactive oxygen species and biomolecules. The ultraviolet A (UVA) radiation component of sunlight (320–400 nm) has been shown to be a source of oxidative stress to skin via generation of reactive oxygen species. We report here that the exposure of human primary skin fibroblasts, FEK4, to UVA radiation causes an immediate release of "free" iron in the cells via proteolysis of Ft. Within minutes of exposure to a range of doses of UVA at natural exposure levels, the binding activity of IRP-1, as well as Ft levels, decreases in a dose-dependent manner. This decrease coincides with a significant leakage of the lysosomal components into the cytosol. Stabilization of Ft molecules occurs only when cells are pretreated with lysosomal protease inhibitors after UVA treatment. We propose that the oxidative damage to lysosomes that leads to Ft degradation and the consequent rapid release of potentially harmful "free" iron to the cytosol might be a major factor in UVA-induced damage to the skin.**

In humans, prolonged sunlight exposure is associated with various pathological states, which include erythema, cataract, skin aging, and cancer. The ultraviolet A (UVA, 320–400 nm) is a major component of sunlight that generates a severe oxidative stress in cells via interaction with intracellular chromophores (1). Singlet oxygen and hydrogen peroxide ($H_2O_2$) are thought to be the most important reactive oxygen species generated intracellularly by UVA, promoting biological damage in exposed tissues via iron-catalyzed oxidative reactions (2). It has been shown that physiologically relevant doses of UVA induce lipid peroxidation in membranes of human primary fibroblasts and keratinocytes via pathways involving iron and singlet oxygen (2–4). Indeed, iron "at" or "near" strategic targets, e.g., cell membranes, can undergo redox cycling by reacting sequentially with one-electron reductants and oxidants, thereby generating toxic oxidants such as hydroxyl radical and lipid-derived alkoxyl and peroxyl radicals and can elicit biological damage (5, 6). Most of the iron that is not metabolized is stored in ferritin (Ft). Ft is a ubiquitously expressed iron-storage protein that forms a hetero-oligomeric protein shell composed of 24 Ft light- (L, 19 kDa) and heavy- (H, 21 kDa) chain subunits (7, 8). Up to 4,500 iron atoms can be sequestered in Ft as a crystalline core of ferric ions ($Fe^{3+}$; ref. 9). This form of storage is thought to protect iron from reduction (10). However, iron is required for numerous metalloenzymes and the synthesis of heme, and little is known about how iron gets from the intracellular stores to the biochemical systems involved in biosynthesis. It is generally assumed that there is a small intracellular pool of "free" iron that is accessible to permeant chelators and comprises the cellular iron that is metabolically and catalytically reactive (10, 11). This pool of reactive "free" iron is sensed by the cytosolic iron-regulatory proteins 1 and 2 (IRPs), which function as regulators of iron uptake and distribution processes in mammalian cells. Indeed, the specific interactions of particular hairpin structures, called iron-responsive elements (IREs), present in the respective mRNAs of genes of the iron-storage (Ft) and iron-uptake [transferrin receptor (TfR)] proteins with the cytosolic IRPs, regulate the level of reactive "free" iron in cells. An increase in iron supply will cause inactivation of IRP-1 and degradation of IRP-2, leading to the induction of Ft mRNA translation and degradation of TfR mRNA, resulting in decreased levels of intracellular "free" iron. Conversely, under conditions of iron deprivation, IRPs bind to IREs, leading to inhibition of Ft mRNA translation and induction of TfR protein synthesis (12–14).

IRP-1, which may itself act as a potentially important iron/citrate carrier (15), is susceptible to oxidative inactivation of RNA binding *in vitro* and *in vivo* (16, 17). By contrast, menadione, nitric oxide, and $H_2O_2$ (18, 19) activate IRP-1 RNA-binding activity and, as a result, lead to a coordinated decrease of Ft synthesis and induction of TfR expression. On the other hand, Cairo *et al.* (20) have observed that in cell-free systems, $H_2O_2$ cannot directly modify IRP-1 but instead can cooperate with superoxide and down-regulate IRP-1 activity. The authors have further demonstrated that down-regulation of IRP-1 occurs also *in vivo* in liver tissue of the rats subjected to ischemia reperfusion or phorone, a glutathione-depleting agent (21, 22).

The cytoprotective role of Ft remained largely hypothetical until it was shown that cells overexpressing this protein are more resistant to oxidative injury (10, 23, 24). However, it has also been suggested that Ft could be a potentially hazardous molecule under pathological conditions, because iron can be reductively released from Ft during oxidative stress (25–30). However, definitive identification of the reductant(s) that operate(s) within the intact cell has not been accomplished. Iron is also liberated from Ft as a consequence of normal turnover in lysosomal compartments, where it is recycled for heme synthesis (31, 32). This source of low molecular-weight iron has recently been shown to be active in the cell-damaging processes caused by oxidative stress promoting lysosomal rupture and release of potent hydrolytic enzymes to the cytosol (33–35).

Oxidative stress can also affect iron homeostasis via activation of the heme-catabolizing enzyme, heme oxygenase-1 (HO-1). UVA radiation induces strong transcriptional activation of the HO-1 gene in human primary skin fibroblasts (36), which eventually (i.e., in 1 to 2 days) leads to a HO-1-dependent increase in Ft (37) and a consequent lowering of the prooxidant state of the cells (38). However, the molecular

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Abbreviations: Ft, ferritin; IRP, iron-regulatory protein; UVA, ultraviolet A; TfR, transferrin receptor; IRE, iron-responsive element; HO-1 and –2, heme oxygenase-1 and -2; H, heavy; L, light.

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mechanisms leading to enhanced Ft synthesis under UVA-mediated oxidative stress have not been fully elucidated. Furthermore, the exact behavior of the IRP-1 in UVA-mediated oxidative stress remains to be determined. We therefore sought to investigate in detail the pattern of cellular iron metabolism after exposure of the human skin fibroblast cell line, FEK4, to UVA and to further explore the cascade of events that leads to the eventual increase in intracellular Ft level after UVA radiation.

## MATERIALS AND METHODS

**Chemicals.** All the protease inhibitors and chemicals were from Sigma , except Lactacystin, MG-132, and chymotrypsin substrate (Suc-Gly-Gly-Phe-*p*NA), which were from Calbiochem, Pefabloc from Boehringer Mannheim, protein G-Sepharose from Pharmacia, Desferal from CIBA–GEIGY, and Lysosensor DND-189 and calcein-AM from Molecular Probes. Cell culture materials were from Life Technologies (Paisley, Scotland), except FCS, which was from PAA Laboratories (Teddington, U.K.) and PBS, which was from Oxoid (Basingstoke, UK).

**Cell Culture and Irradiation.** FEK4 cells ($3 \times 10^5$; passages 12 to 14) grown for 3 days in 10-cm plastic plates were cultured to 80% confluency as described (39). Before irradiation, medium was removed, and cells were washed thoroughly with PBS and covered in PBS containing $Ca^{2+}$ and $Mg^{2+}$ (0.01% each). Monolayers of cells were irradiated at 100-, 250-, and 500-kJ/m$^2$ UVA doses. The UVA doses were measured by using an IL1700 radiometer (International Light, Newburyport, MA). The irradiations were carried out in an air-conditioned room (18°C), and the variation of temperature in irradiated plates was monitored with a control plate (containing PBS) linked to a digital thermometer. This measurement revealed that during irradiation, the overall temperature of plates varies between 24 and 26°C. Control fibroblasts were treated in the same manner, except that they were not irradiated. All irradiations were with a broad-spectrum Sellas 4kW UVA lamp (Sellas, Germany). This lamp emits primarily UVA radiation (significant emission in the range of 350–400 nm) and some near-visible radiation longer than 400 nm. The incident dose rate was 500W/m$^2$.

**Calcein Loading and Intracellular Chelatable Iron Estimation.** The method used to estimate the level of intracellular chelatable iron was modified from Epsztejn *et al.* (40). This method is based on total dequenching of the sector of calcein fluorescence that is quenched by bound iron. After irradiation with a UVA dose of 250 kJ/m$^2$, cells were loaded with 0.05 $\mu$M calcein-AM in 1 ml Earle's minimum essential media (containing 20 mM Hepes, pH 7.3) for 15 min at 37°C. Then cells were washed with PBS (containing 20% FCS) and resuspended in 10 mM Hepes buffer containing diethyltriamine-pentaacetic acid (2 mM). The cell suspension was transferred to a thermostatically controlled cuvette (with stirring), and calcein fluorescence (excitation 480 nm, emission 517 nm, 10 nm slit width) was recorded on a Kontron (SFM 25, Zurich) spectrofluorimeter. The level of intracellular calcein-bound iron was determined by the increase in fluorescence produced by the addition of the highly permeable iron chelator, salicaldehyde isonicotinoyl hydrazone (kindly provided by P. Ponka, Lady Davis Institute for Medical Research, Montreal, Canada). To establish the relationship between fluorescence change and intracellular chelatable iron concentrations, calibrations were performed in nonirradiated FEK4 cells. After treatment with ionophore A23187 (10 $\mu$M), ferrous ammonium sulfate (0.1 $\mu$M) was added cumulatively and the corresponding change in fluorescence determined.

**IRP/IRE Bandshift Assays.** For the time course experiments, the cytoplasmic extracts were first prepared at the indicated time points after UVA irradiation according to Mullner *et al.* (41) and then probed with gel-purified $^{32}$P-labeled wild-type or IRP-1-specific RNAs (kindly provided by L. Kuhn; see ref. 42) in the presence of 5 mg/ml heparin. RNA–protein complexes were resolved on a 6% nondenaturing gel and processed for autoradiography. Quantitation of the IRP/IRE signals was carried out by photodensitometry (Bio-Rad scanner, model GD-670) of the autoradiographs, by using MOLECULAR ANALYST version 1.4.1 software.

**Cytosolic Aconitase Activity.** Cytosolic extracts devoid of mitochondrial aconitase were prepared as in Giordani *et al.* (43). The cytosolic aconitase activity was determined by measuring the decrease of *cis*-aconitate as assessed by spectrophotometric absorbance at 240 nm as in Drapier and Hibbs (44). Specific activity ($\mu$mol of substrate converted/mg protein/min at 25°C) was calculated by assuming an extinction coefficient of 3.41 mM$^{-1}$ cm$^{-1}$ for *cis*-aconitate, based on the data of Henson and Cleland (45).

**Metabolic Labeling in Cultured Cells.** Eighty percent confluent FEK4 cells were starved for 15 min in methionine-free minimum essential media supplemented with 5% FCS followed by [$^{35}$S]methionine labeling for 4 h. The cells were then irradiated at the indicated doses as described above (the control fibroblasts were treated as above, except that they were not irradiated) and lysed in immunoprecipitation buffer (50 mM Tris, pH 7.4/150 mM NaCl/10 mM EDTA/1% Triton X-100/1% SDS/20 $\mu$g/ml leupetin/100 $\mu$g/ml chymostatin/1 mM PMSF/1 $\mu$g/ml pepstatin/10 $\mu$g/ml E64/50 $\mu$g/ml Pefabloc). Equal amounts of labeled proteins were immunoprecipitated by using either anti-human Ft polyclonal or human polyclonal anti-HO-2 (from this laboratory) antibodies and Protein G-Sepharose and loaded on a 12% SDS-polyacrylamide gel. After drying, the gel was exposed for autoradiography. For the determination of the rate of Ft synthesis, cells were labeled only for 2 h (15 min starvation in methionine-free media and 1 h 45 min labeling) before extraction and lysis.

**Protease Inhibitor Treatments.** For all the specified treatments, cells were first treated overnight with the specified protease inhibitors in conditioned media, and then a fresh solution was added to the labeling media. The same specific protease inhibitors were present both during irradiation and in the lysis buffer at the required concentrations.

**Lysosome Localization.** Eighty percent confluent FEK4 cells grown on coverslips (inside Petri dishes) were first irradiated with UVA at the indicated doses and then incubated for 30 min with media containing 1 $\mu$M lysosensor probe DND-189. Next, the loading media was replaced with fresh medium, and the coverslips were mounted on glass slides. The lysosomal integrity was monitored under an epifluorescence microscope (absorbance at 443 nm and emission at 505 nm).

**Assay for Chymotrypsin Activity in Cytoplasmic S-100 Supernatants.** After irradiation of 80% confluent cells with a UVA dose of 250 kJ/m$^2$, cytosolic S-100 fractions (devoid of intact organelles) were prepared according to the method of Dignam *et al.* (46), with the exception that in all buffers DTT and chymostatin were omitted. The cytosolic S-100 fractions were used for the determination of chymotrypsin activity against the chymotrypsin-specific chromogenic substrate (Suc-Gly-Gly-Phe-*ρ*NA) as described by Achstetter *et al.* (47). Before assay, the cytosolic S-100 fractions from control and UV-irradiated cells were incubated either for 15 min at 37°C to allow proteases to become fully active or in the presence of 200 $\mu$g/ml chymostatin to block completely the chymotrypsin activity (used as negative controls). Specific activity was calculated as nanomoles 4-nitriloaniline liberated/min/mg protein assuming a molar absorption coefficient of 9,500 liters mol$^{-1}$ cm$^{-1}$ for 4-nitriloaniline at 405 nm, based on the data of Erlanger *et al.* (48).

**Statistical Methods.** Results are expressed as the mean ± one standard deviation. Significant differences ($p < 0.05$) were determined by either paired or unpaired *t* test after one-way analysis of variance.

## RESULTS AND DISCUSSION

We followed the kinetics of iron mobilization by RNA-bandshift assay in primary human skin fibroblasts (FEK4) after UVA irradiation. The level of interaction of an oligonucleotide containing the human IRP-1-specific IRE motif with the IRP-1 present in cytoplasmic extracts of the cells was used to estimate the level of intracellular free iron in FEK4 cells (42). The UVA doses used were 100, 250, and 500 kJ/m$^2$, which are equivalent to the amount the surface of skin would be exposed to on a summer day around noon at a northern latitude of 30–35° (49). A moderate dose of 250 kJ/m$^2$ UVA radiation inactivates 15 ± 5% of an FEK4 cell population. The highest dose used in this study (500 kJ/m$^2$) inactivates more than 50% of the cells (39). The results (Fig. 1*A*) showed that immediately after UVA irradiation, IRP-1-binding activity drops in a dose-dependent manner (Fig. 1*A*, lanes 2–4). The immediate decrease in IRP-1-binding activity in cells irradiated with UVA doses of 100 and 250 kJ/m$^2$ correlated with a reciprocal increase in the aconitase activity of the IRP-1 (Fig. 1*C*), which is known to occur in response to the increased availability of intracellular iron and as a result of the assembly of the intact cubane 4Fe–4S cluster in the IRP-1 protein (50). These results strongly suggested the release of "free" transit iron in the cytosol. At a higher dose of 500 kJ/m$^2$, the aconitase activity was significantly lower than the control cells, presumably reflecting severe damage to the protein. The decrease in IRP-1-binding activity returned to around control value for cells irradiated with low to moderate doses of 100 and 250 kJ/m$^2$ UVA within the 6 h after irradiation (data not shown). At a higher dose of 500 kJ/m$^2$, the drop in IRP-1/IRE signals was sustained for at least 24 h after irradiation (result not shown), suggesting that in the case of severe oxidative damage, iron homeostasis could be severely perturbed, and this is reflected by the reduced cell survival at higher doses of UVA radiation (39). A similar dose-dependent decrease in the binding activity of IRPs was observed when the wild-type IRE motif (which recognizes both human IRP-1 and IRP-2; see ref. 42) was used in bandshift assays after UVA irradiation of FEK4 cells (data not shown). To ascertain that the immediate decrease in IRP-1-binding activity after UVA irradiation of cells is not caused by its eventual degradation, we performed immunoblotting analysis in the cytoplasmic extracts of UVA-irradiated cells with an antibody specific to human IRP-1. The result (not shown) revealed that the cellular content of IRP-1 remained similar, regardless of doses of UVA applied, inconsistent with severe UVA-mediated degradation of IRP-1 protein. In addition, before IRP/IRE bandshift assay, the cytoplasmic extracts prepared after 0-, 2-, and 6-h irradiation with UVA doses of 100, 250, and 500 kJ/m$^2$ were incubated *in vitro* with 2% $\beta$-mercaptoethanol ($\beta$-ME) to monitor the level of total IRP-1 in the cells. The results (not shown) revealed that *in vitro* reduction with $\beta$-ME recovered mostly the IRP-1 activity in extracts from UVA-treated cells, although to a lesser extent than in extracts prepared immediately after irradiation. To further reinforce our hypothesis that the UVA-mediated decrease in IRP-1-binding activity reflects the presence of "free" transit iron in the cells, we preincubated the cells with the strong iron chelator Desferal (Desferrioxamine) for 18 h before UVA irradiation with a UVA dose of 250 kJ/m$^2$. The results (Fig. 2) showed that in agreement with our hypothesis, Desferal pretreatment markedly reduced the inactivation of IRP-1 by UVA radiation. Finally, we measured the level of free chelatable intracellular iron immediately after irradiation (250 kJ/m$^2$) of FEK4 cells by the calcein fluorescence dequenching assay developed by Cabantchik and coworkers (40). As is summarized in Table 1, the level of free chelatable iron in UVA-irradiated cells (250 kJ/m$^2$) increased 2.7-fold when compared with unirradiated controls. Interestingly, treatment of cells with Desferal before UVA irradiation almost completely abolished the level of free chelatable iron in the cells (Table 1). Taken together, these findings are consistent with the notion that UVA induces immediate release of free "transit" iron in the cells.

We then verified whether the iron release caused by UVA in FEK4 cells originates from Ft, which is a major source of iron in the cells. The results (Fig. 3) showed that the immediate decrease in IRP-1 activity after UVA irradiation coincided with a significant and dose-dependent decrease in Ft levels monitored by immunoprecipitation of the same extracts with human polyclonal Ft antibody (Fig. 3*A*). The recovery of Ft synthesis after irradiation with a moderate dose of 250 kJ/m$^2$ (Fig. 3*B*) coincided with that of the IRP-1/IRE signal (Fig. 2), which returned to around the control value within 6 h of irradiation (Fig. 3*B*, lane 6). On the other hand, the rate of synthesis of the HO-2 protein (the constitutive isozyme of HO-1 protein whose expression is not altered after UVA

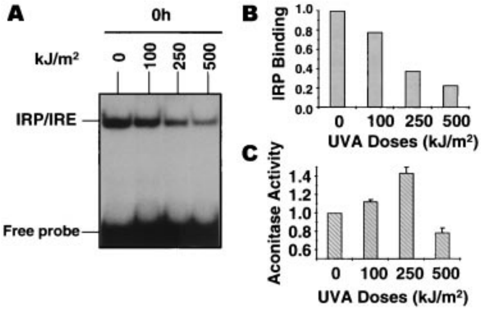


FIG. 1. (*A*) Modulation of IRP-1/IRE-binding activity 0 h after irradiation of human FEK4 fibroblasts with UVA doses of 100, 250, and 500 kJ/m$^2$. The positions of the IRP/IRE complexes and of excess free probe are indicated. (*B*) The IRP/IRE signals of irradiated cells (from *A*) were quantified and their relative intensities normalized against that of nonirradiated control (in *A*) and expressed as a fold increase in IRP-1-binding activity above control value as a function of UVA dose. (*C*) The effect of UVA exposure on aconitase activity in IRP-1 (*B*) after UVA irradiation in FEK4 cells exposed to doses up to 250 kJ/m$^2$. The aconitase activity of irradiated samples (mean ± SD) were expressed as a fold increase in aconitase activity compared with that of unirradiated controls (0 kJ/m$^2$) as a function of UVA doses. $n$ = 4–6 independent experiments in which three measurements have been performed.

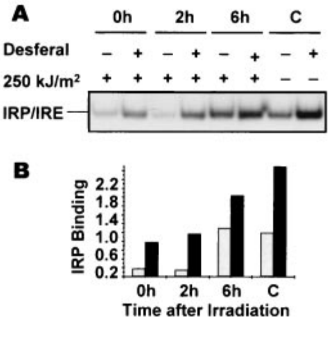


FIG. 2. (*A*) Effect of Desferal pretreatment (100 $\mu$M, for 18 h) on modulation of IRP-1-binding activity after UVA irradiation of FEK4 cells with a dose of 250 kJ/m$^2$. The treated samples are shown as (+). The nontreated samples are shown as (–). (*B*) The IRP/IRE signals (from *A*) were quantified and their relative intensities normalized against that of the nonirradiated control cells (without Desferal pretreatment) and then expressed as a fold increase in IRP-1-binding activity above control value as a function of time after irradiation (h). (*C*) The controls that were not irradiated.

Table 1. Effect of UVA irradiation (250 kJ/m$^2$) with/without pretreatment with Desferal (100 $\mu$M, 18 h) on the concentration of chelatable iron in FEK4 cells. Measures were performed immediately (0 h) after irradiation

| Sample | Chelatable iron ($\mu$M) |
|---|---|
| Unirradiated control | 0.057 $\pm$ 0.024 |
| UVA | 0.156 $\pm$ 0.014* |
| Unirradiated control + Desferal | 0.000 $\pm$ 0.000* |
| UVA + Desferal | 0.022 $\pm$ 0.022 |

Results expressed as means $\pm$ SD of triplicate analyses of $n$ = 3–6 independent experiments. Statistical analyses were made by using a paired $t$ test. *, Significantly different from unirradiated control ($p <$ 0.05 level).

radiation) monitored during the same period remained unchanged (Fig. 3*C*), suggesting that the decrease in Ft level after UVA radiation is not a general effect on translation. These results, taken together with the observation that Desferal pretreatment, which is known to down-regulate Ft synthesis, prevents the UVA-mediated IRP-1 inactivation (Fig. 2), are consistent with the hypothesis that the immediate release of "free" iron after UVA irradiation arises from the degradation of Ft, a major source of iron in the cells.

It is known that Ft synthesis is controlled at the translational level via IRPs, which, during iron deprivation, bind to IRE elements in untranslated regions of Ft H- and L-mRNAs and thereby suppress their translation. Inactivation of IRPs by high iron concentrations prevents the IRP/IRE interactions and therefore allows the efficient translation of Ft mRNAs. However, recent studies with rodents have shown that after oxidative stress, the level of accumulation of H- and L- Ft mRNAs is also increased, implying control at the transcriptional level (21, 22). To assess the importance of transcriptional mechanisms in increasing Ft synthesis in our model, the effect of UVA on the level of H- and L-subunit mRNAs was analyzed by Northern blotting. The results (not shown) revealed a low (up to 1.5-fold) but significant ($p$ < 0.001) increase in the steady-state levels of both transcripts after UVA irradiation with a peak at 6 h after irradiation. Therefore it appears that the level of induction of Ft synthesis after UVA irradiation is controlled at both the transcriptional and translational levels, although the translation effect is almost certainly predominant.

Although the iron-scavenging property of Ft is well known (see Introduction), it has been shown that the iron content of Ft can be readily mobilized under pathological conditions or in cells exposed to reducing conditions or free radicals (25–30).

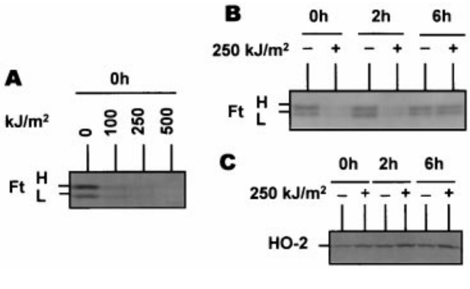


FIG. 3. (*A*) Dose-dependent degradation of Ft after (0 h) UVA irradiation of FEK4 fibroblasts as monitored by immunoprecipitation of $^{35}$S-labeled FEK4 cells with human Ft polyclonal antibody. Lane 1 represents the nonirradiated control and lanes 2–4 represent samples irradiated with UVA doses of 100, 250, and 500 kJ/m$^2$, respectively. (*B*) Ft biosynthesis after UVA irradiation of FEK4 cells. (*C*) HO-2 biosynthesis after UVA irradiation of Fek4 cells. In *B* and *C*, the rate of protein (Ft and HO-2, respectively) synthesis was followed by $^{35}$S-labeling 2 and 6 h after UVA irradiation of cells with a dose of 250 kJ/m$^2$ (+). Nonirradiated controls are shown as (–). In the 0-h time point, cells were labeled before UVA irradiation.

To elucidate whether UVA-mediated iron release from Ft in FEK4 cells is occurring as a direct result of damage to Ft protein shells or as a consequence of proteolysis involving either the lysosomal compartments or proteasomes, we used the following strategies: the level of Ft was monitored by immunoprecipitation after UVA irradiation (250 kJ/m$^2$) of cells that had been treated either with one of a series of protease inhibitors (Pepstatin, E64-d, Leupeptin, Chymostatin, and Calpeptin) or with the highly specific proteasome inhibitors, Lactacystin and MG132 (51, 52). To determine the optimal concentration of protease inhibitors, we performed a dose response for each protease inhibitor up to lethal concentrations (results not shown). The results (Fig. 4) showed that only Chymostatin and Leupeptin pretreatment (or a combination of the two) prevented the degradation of Ft after UVA treatment (Fig. 4*A*), so that the release of iron from Ft is mediated via a proteolytic event related to lysosomal proteases rather than proteasomal trypsin-like or chymotrypsin-like activities (Fig. 4*C*). Previous studies also provide evidence that Chymostatin and Leupeptin inhibit specifically the protease(s) responsible for Ft breakdown in the lysosomes (31, 53). However, the inhibition of lysosomal function by weak bases such as Chloroquine or ammonium chloride failed to stabilize the level of Ft protein after UVA treatment, so that the proteolysis is independent of its uptake by lysosomes (Fig. 4*D*).

Two questions have arisen from our observations: firstly, what is the exact process leading to the proteolytic degradation of Ft in the cytosol, and secondly, how are the lysosomal proteases involved in this process? Several studies have shown that during cellular injury by oxidative stress, lysosomal membranes could be destabilized through lipid peroxidation promoting lysosomal rupture and release of potent hydrolytic enzymes to the cytosol (35, 54–57). UVA is a membrane-damaging agent, and it has been shown that iron and singlet oxygen contribute to peroxidation of human skin fibroblast membranes (2). Taken together, these results suggest that UVA may also trigger the peroxidation of lysosomal membranes, leading to the destabilization of the organelles and the leakage of potentially harmful proteolytic enzymes to the cytosol. We therefore tested the involvement of lysosomal damage in proteolytic degradation of Ft after UVA irradiation of FEK4 cells. The evaluation of the stability of lysosomal membranes after UVA irradiation of FEK4 cells was performed by means of an acidotropic fluorescent probe, Lysosensor green DND-189, which accumulates in acidic organelles

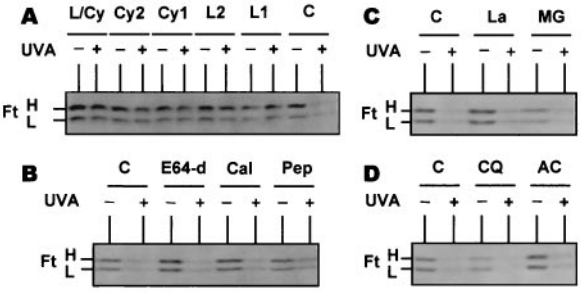


FIG. 4. Effect of protease inhibitors on Ft degradation after UVA irradiation of FEK4 cells. $^{35}$S-labeled FEK4 cells that were pretreated overnight with the specified drug(s) were irradiated at 250 kJ/m$^2$ in the presence of inhibitors. Immediately after UVA radiation, cells were lysed and immunoprecipitated with human polyclonal Ft antibody. The drugs used were: (*A*) 20 (L1) and 40 $\mu$g/ml Leupeptin (L2), 100 (Cy1) and 200 $\mu$g/ml Chymostatin (Cy2), or the combination of 40 $\mu$g/ml Leupeptin and 200 $\mu$g/ml Chymostatin (L/Cy); (*B*) 50 $\mu$g/ml E64-d and Calpeptin (Cal) or 1 $\mu$g/ml Pepstatin (Pep); (*C*) 100 $\mu$M Lactacystin (La) or MG132 (MG); (*D*) 50 $\mu$g/ml Chloroquine (CQ) and 30 mM ammonium chloride (AC). The irradiated samples are shown as (+). The nonirradiated controls are shown as (–). In *A–D*, "C" represents the controls that were not treated with drugs.

as the result of protonation and exhibits a pH-dependent increase in fluorescent intensity on acidification. Fig. 5*A* shows the distribution of the fluorescent dye within the intact lysosomes of living FEK4 cells. After UVA irradiation, the intensity of fluorescent dye decreased in a dose-dependent manner (Fig. 5 *B–D*), reflecting the leakage of lysosomal membranes and consequently the release of fluorescent dye into the cytosol. At a high dose of 500 kJ/m$^2$, almost no fluorescent vesicles were observed in the cells (Fig. 5*D*). This phenomenon was reversible for the cells irradiated with the doses of 100 and 250 kJ/m$^2$, where the cells returned to normal within 6 h postirradiation time (results not shown). However, at a higher dose of 500 kJ/m$^2$, damage to the lysosomal membrane was only partially recovered around 24 h after UVA irradiation (result not shown). We also measured the level of activity of lysosomal protease, chymotrypsin, in the cytosolic fractions of cells (devoid of intact membrane organelles) extracted immediately after irradiation with a moderate dose of 250 kJ/m$^2$. Because Chymostatin has been shown to specifically block lysosomal chymotrypsin activity, we have used a substrate that is specific to this form of protease (see ref. 47). As is shown in Table 2, the cytoplasmic fraction of UVA-irradiated cells contains significantly higher amounts of chymotrypsin activity (up to 3-fold) than unirradiated control cells. In a related study, we have also observed that the level of activity of lysosomal cathepsin B and L proteases in cytosolic fractions of UVA-irradiated FEK4 cells is 2-fold higher than in unirradiated control cells (C. Waltner & R.M.T., unpublished data).

A

B

C

D

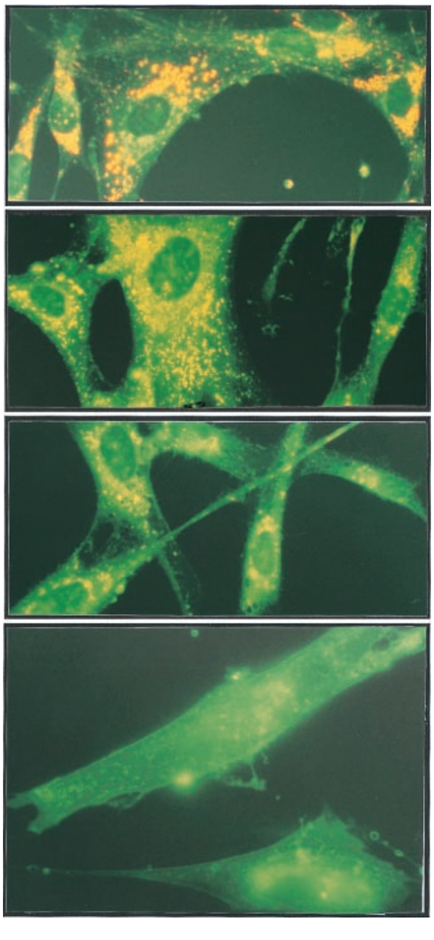

FIG. 5. UVA-mediated dose-dependent damage to lysosomal membranes in FEK4 cells as monitored after 30-min treatment with the acidotropic lysosomal dye under an epifluorescence microscope. (*A*) Nonirradiated control cells. (*B*) Cells irradiated with 100 kJ/m$^2$. (*C*) Cells irradiated with 250kJ/m$^2$. (*D*) Cells irradiated with 500 kJ/m$^2$. (*A–C*, ×400; *D*, ×600, in presence of immersion oil.)

Table 2. Chymotrypsin activity in cytoplasmic extracts (S100 fraction) of FEK4 cells after 250 kJ/m$^2$ UVA irradiation. Activity is expressed as nanomols 4-nitroaniline liberated per minute per milligram protein

| Sample | 4-nitroaniline released |
|---|---|
| Unirradiated control | 0.43 ± 0.10 |
| UVA | 1.36 ± 0.13 |

Results expressed as means ± SD of triplicate analyses of $n = 6$ independent experiments. Statistical analyses were made using a paired *t* test. *, Significantly different from unirradiated control ($p <$ 0.05 level).

These results strongly suggest that the leakage of lysosomal proteases into the cytosol of irradiated cells may be the origin of UVA-mediated degradation of Ft.

To ascertain whether down-regulation of IRP-1 after UVA radiation of FEK4 cells was entirely related to the Ft-mediated iron release, we monitored the level of IRP-1-binding activity in cells that were pretreated with Leupeptin and Chymostatin before UVA radiation. The results (not shown) revealed that treatment of cells with the protease inhibitors Leupeptin and Chymostatin before UVA irradiation (250 kJ/m$^2$) only partially recovers the initial UVA-mediated reduction in IRP-1 binding activity, indicating that other sources of iron in addition to Ft are responsible for the decrease in binding after UVA irradiation. One such source is the heme that is released from microsomal hemoproteins immediately after UVA irradiation of FEK4 cells (58).

Our current findings provide new information on the molecular mechanisms underlying the UVA-mediated increase in Ft levels. It appears that a combination of "early" degradation of Ft together with "early" microsomal heme release and the "intermediate" HO-1-mediated heme breakdown (36) all contribute to the iron pool that is responsible for the increased Ft synthesis after UVA irradiation of FEK4 cells. When the complete kinetics of Ft synthesis after UVA radiation are monitored by immunoprecipitation (data not shown), it can be further demonstrated that, beyond 6 h postirradiation, the rate of Ft synthesis exceeds that of the control and at 24 h after irradiation, this rate is 2- to 3-fold higher than in nonirradiated controls. The latter results are in agreement with the previous finding from Vile and Tyrrell (37) that the level of Ft increases up to 2-fold 24 to 48 h after UVA irradiation of FEK4 cells.

In summary, we show here that UVA radiation leads to an immediate increase in the free available iron via degradation of the Ft molecule. The consequent down-regulation of IRP-1 stimulates Ft synthesis at both the transcriptional and translational levels and eventually leads to an enhanced level of the iron storage protein. We also provide the first evidence that the Ft degradation that occurs after UVA radiation originates from destabilization of lysosomal membranes and the subsequent leakage of proteolytic enzymes from these organelles. The consequent release of potentially harmful free "transit" iron within the cells will clearly exacerbate the damaging effects of photoperoxidation and is likely to be of central importance to both the reversible and degenerative damage to the skin after exposure to solar UV. Furthermore, we show that UVA leads to a rapid degradation of the major iron-storage protein, Ft. The lack of this critical iron-storage protein would be expected to further exacerbate the consequence of the UVA-released iron in human cells. The identification of early events that occur in human skin cells as a result of exposure to low levels of UVA irradiation should provide insights into rational approaches to prevent skin damage. The presence of excess iron has also been demonstrated in a variety of skin disorders such as psoriasis (59), venous ulceration (60), and atopic eczema (61). Finally, our findings are entirely in agreement with the studies of Bisset *et al.* (62, 63) reporting a

3-fold increase in the iron content of human epidermis from sun-exposed areas as compared with nonexposed body sites.

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1. Tyrrell, R. M. (1996) *BioEssays* **18,** 139–148.
2. Vile, G. F. & Tyrrell, R. M. (1995) *Free Radical Biol. Med.* **18,** 721–730.
3. Morliere, P., Moysan, A., Santus, R., Huppe, G., Maziere, J.-C. & Ertret, L. (1991) *Biochim. Biophys. Acta* **1084,** 261–268.
4. Punnonen, K., Jansen, C. T., Puntala, A. & Ahotoupa, M. (1991) *J. Invest. Dermatol.* **96,** 255–259.
5. Aust, S. D., Morehouse, L. A. & Thomas, C. E. (1985) *J. Free Radicals Biol. Med.* **1,** 3–25.
6. Halliwell, B. & Gutteridge, J. M. C. (1992) *FEBS Lett.* **307,** 108–112.
7. Munro, H. N. & Linder, M. C. (1978) *Physiol. Rev.* **58,** 317–396.
8. Theil, E. C. (1987) *Annu. Rev. Biochem.* **56,** 289–315.
9. Richter, G. W. (1978) *Am. J. Pathol.* **91,** 363–404.
10. Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J. W. & Vercellotti, G. M. (1992) *J. Biol. Chem.* **267,** 18148–18153.
11. Breuer, W., Epsztejn, S. & Cabantchik, Z. I. (1996) *FEBS Lett.* **382,** 304–308.
12. Klausner, R. D., Rouault, T. A. & Harford, J. B. (1993) *Cell* **72,** 19–26.
13. Kuhn, L. C. (1994) in *Bailliere's Clinical Haematology*, ed. Hershko, C. (Bailliere Tindall, London), pp. 763–785.
14. Guo, B., Brown, F. M., Phillips, J. D., Yu, Y. & Leibold, E. A. (1995) *J. Biol. Chem.* **270,** 16529–16535.
15. Beinert, H. & Kennedy, M. C. (1993) *FASEB J.* **7,** 1442–1449.
16. Hentze, M. W., Rouault, T. A., Harford, J. B. & Klausner, R. D. (1989) *Science* **244,** 357–359.
17. Mullner, E. W., Rothenberger, S., Muller, A. M. & Kuhn, L. C. (1992) *Eur. J. Biochem.* **208,** 597–605.
18. Martins, E. A. L., Robalinho, R. L. & Meneghini, R. (1995) *Arch. Biochem. Biophys.* **316,** 128–134.
19. Pantopoulos, K. & Hentze, M. W. (1995) *EMBO J.* **14,** 2917–2924.
20. Cairo, G., Castrusini, E., Minotti, G. & Bernelli-Zazzera, A. (1996) *FASEB J.* **10,** 1326–1335.
21. Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A. & Bernelli-Zazzera, A. (1995) *J. Biol. Chem.* **270,** 700–703.
22. Tacchini, L., Recalcati, S., Bernelli-Zazzera, A. & Cairo, G. (1997) *Gastroenterology* **113,** 946–953.
23. Cermak, J., Balla, J., Jacob, H. S., Balla, G., Enright, H., Nath, K. & Vercellotti, G. M. (1993) *Cancer Res.* **53,** 5308–5313.
24. Lin, F. & Girotti, A. W. (1997) *Arch. Biochem. Biophys.* **346,** 131–141.
25. Reif, D. W. (1992) *Free Radical Biol. Med.* **12,** 417–427.
26. Aust, S. D., Chignell, C. F., Bray, T. M., Kalyanaraman, B. & Mason, R. P. (1993) *Toxicol. Appl. Pharmacol.* **120,** 168–178.
27. Bolann, B. J. & Ulvik, R. J. (1990) *Eur. J. Biochem.* **193,** 899–904.
28. Bando, Y. & Aki, K. (1990) *Biochem. Biophys. Res. Commun.* **168,** 389–395.
29. Aubailly, M., Santus, R. & Salmon, S. (1991) *Photochem. Photobiol.* **54,** 769–773.
30. Oteiza, P. I., Kleinman, C. G., Demasi, M. & Bechara, E. J. H. (1995) *Arch. Biochem. Biophys.* **316,** 607–611.
31. Vaisman, B., Fibach, E. & Konijn, M. (1997) *Blood* **90,** 831–838.
32. Radisky, D. C. & Kaplan, J. (1998) *Biochem. J.* **336,** 201–205.
33. Ollinger, K. & Brunk, U. T. (1995) *Free Radical Biol. Med.* **19,** 565–574.
34. Zhang, H., Dalen, H., Ollinger, K. & Brunk, U. T. (1995) *Free Radical Biol. Med.* **19,** 813–822.
35. Roberg, K. & Ollinger, K. (1998) *Am. J. Pathol.* **152,** 1151–1156.
36. Keyse, S. M. & Tyrrell, R. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 99–103.
37. Vile, G. F. & Tyrrell, R. M. (1993) *J. Biol. Chem.* **268,** 14678–14681.
38. Vile, G. F., Basu-Modak, S., Waltner, C. & Tyrrell, R. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2607–2610.
39. Tyrrell, R. M. & Pidoux, M. (1988) *Photochem. Photobiol.* **47,** 405–412.
40. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W. & Cabantchik, Z. I. (1997) *Anal. Biochem.* **248,** 31–40.
41. Mullner, E. W., Neupert, B. & Kuhn, L. C. (1989) *Cell* **58,** 373–382.
42. Henderson, B. R., Menotti, E. & Kuhn, L. C. (1996) *J. Biol. Chem.* **271,** 4900–4908.
43. Giordani, A., Morliere, P., Djavaheri-Mergny, M. & Santus, R. (1998) *Photochem. Photobiol.* **68,** 309–313.
44. Drapier, J. C. & Hibbs, J. B. (1986) *J. Clin. Invest.* **78,** 790–797.
45. Henson, C. P. & Cleland, W. W. (1967) *J. Biol. Chem.* **242,** 3833–3838.
46. Dignam, J. D., Lebolitz, R. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
47. Achstetter, T., Ehmann, C. & Wolf, D. H. (1981) *Arch. Biochem. Biophys.* **207,** 445–454.
48. Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95,** 271–278.
49. Frederick, J. E. & Lubin, D. (1988) *Photochem. Photobiol.* **47,** 571–578.
50. Haile, D. J., Rouault, T. A., Tang, C. K., Chin, J. & Harford, J. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7536–7540.
51. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J. & Schreiber, S. L. (1995) *Science* **268,** 726–731.
52. Tawa, N. E., Jr., Odessey, R. & Goldberg, A. L. (1997) *J. Clin. Invest.* **100,** 197–203.
53. Roberts, S. & Bomford, A. (1988) *J. Biol. Chem.* **263,** 19181–19187.
54. Brunk, U. T., Dalen, H., Roberg, K. & Hellquist, H. B. (1997) *Free Radical Biol. Med.* **23,** 616–626.
55. Voogd, A., Sluiter, W., van Eijk, H. G. & Koster, J. F. (1992) *J. Clin. Invest.* **90,** 2050–2055.
56. Mak, I. T., Misra, H. P. & Weglicki, W. B. (1983) *J. Biol. Chem.* **253,** 13733–13737.
57. Roberg, K. & Ollinger, K. (1998) *J. Histochem. Cytochem.* **46,** 411–418.
58. Kvam, E., Noel, A., Basu-Modak, S. & Tyrrell, R. M. (1999) *Free Radical Biol. Med.* **26,** 511–517.
59. Molin, L. & Wester, P. O. (1973) *Acta Derm. Venereol.* **53,** 473–476.
60. Ackerman, Z., Seidenbaum, M., Loewenthal, E. & Rubinow, A. (1988) *Arch. Dermatol.* **124,** 1376–1378.
61. David, T. J., Wells, F. E. & Sharpe, T. C. (1990) *Br. J. Dermatol.* **122,** 485–489.
62. Bissett, D. L., Chatterjee, R. & Hannon, D. P. (1991) *Photochem. Photobiol.* **54,** 215–223.
63. Bissett, D. L. and McBride, J. F. (1992) *J. Soc. Cosmet. Chem.* **43,** 215–217.