Heme oxygenase-1 mRNA levels increase following exposure of many mammalian cell lines to oxidative stress such as ultraviolet A (UVA) irradiation. Here we demonstrate a 4-fold increase in microsomal heme oxygenase activity and a 40% decrease in microsomal heme content 14 h after treatment of human skin fibroblasts (FEK) with 250 kJ m⁻² of UVA radiation. Paralleling this was a 2-fold increase in ferritin levels that was sustained for at least 46 h after UVA irradiation. Treatment of fibroblasts with the iron chelating agent desferrioxamine, after the UVA-dependent induction of heme oxygenase, prevented the increase in ferritin levels. Treatment of fibroblasts with Sn-protoporphyrin IX (an inhibitor of heme oxygenase) also prevented the effect of UVA radiation on ferritin levels. Thus we conclude that the effect of UVA radiation on ferritin levels is via the heme oxygenase-dependent release of iron from endogenous heme sources. We propose that the increase in ferritin that follows UVA irradiation would decrease intracellular free iron such that iron-catalyzed free radical reactions would be restricted during periods of subsequent oxidative stress.

Many procaryotic and eucaryotic cells synthesize specific proteins in response to oxidative stress. In some cases these stress proteins have been shown to have an antioxidant role, e.g. treatment of Escherichia coli with hydrogen peroxide results in expression of at least 30 proteins including catalase (1). The protein most consistently activated by oxidative stress in a wide variety of eucaryotic cells is the heme degrading enzyme, heme oxygenase-1 (2-5). UVA radiation (320-380 nm), hydrogen peroxide, and glutathione depleting compounds all enhance heme oxygenase-1 mRNA synthesis (6) and result in accumulation of heme oxygenase-1 mRNA in cultured human skin fibroblasts (FEK) and other mammalian cell lines (7).

UVA irradiation of biological molecules gives rise to superoxide and hydrogen peroxide (8-10), species that may be involved not only in cell death but also in the carcinogenic effects of UVA irradiation (11). There is good evidence that the biological damage attributed to superoxide and hydrogen peroxide is dependent on the presence of iron (12-14). It has been proposed that there is a small intracellular pool of free iron that can react with hydrogen peroxide and superoxide, giving rise to the very reactive hydroxyl radical (·OH) via Reactions 1 and 2, and it is the hydroxyl radical that is the initiator of biological damage (15-17).

\[
\begin{align*}
G_0 + \text{Fe}^{2+} & \rightarrow \text{Fe}^{3+} + O_2 \\
\text{Fe}^{3+} + H_2O_2 & \rightarrow \cdot OH + OH^- + \text{Fe}^{2+}
\end{align*}
\]

**REATIONS 1 AND 2**

Intracellularly most of the iron that is not metabolized is sequestered in ferritin as a crystalline core of ferric (Fe³⁺) ions (18). To catalyze oxidative reactions, the iron must first be released from the core. Although superoxide is able to do this, it is very inefficient (19). Thus ferritin is able to restrict the availability of iron to participate in Reaction 1.

It has been shown that a hemin-dependent increase in heme oxygenase protein synthesis activates ferritin mRNA translation in rat fibroblasts (20). Whether this led to an increase in heme oxygenase activity or ferritin levels was not examined. Elevated levels of newly synthesized ferritin would result in an enhancement of cellular iron sequestering capacity that may confer increased resistance to oxidative stress. This study tests the hypothesis that levels of ferritin in human skin fibroblasts are effected by UVA irradiation and that this occurs via heme oxygenase.

**MATERIALS AND METHODS**

All biochemicals were from Sigma except where indicated.

*Cell Culture*- Monolayers of the normal human skin fibroblast line (FEK) were grown to 100% confluence in 15-cm dishes over 7 days in minimum essential media supplemented with penicillin, streptomycin, glutamine, sodium carbonate, and 15% fetal calf serum. At day 7 each dish contained approximately 8 × 10⁶ fibroblasts. For some experiments fibroblasts were grown to 40% confluence over 3 days. Fibroblasts were passaged twice a week and used between passages 9 and 16. Cell culture materials were from Life Technologies (Paisley, Scotland), except fetal calf serum, which was from Biological Industries (Harmek, Israel).

*UVA Irradiation*- Fibroblasts were irradiated with 250 kJ m⁻² of broad spectrum UVA light using a Uveon 3000 lamp (Mutzhas, Munich, Germany). The UVA dose was measured using an IL 1700 radiometer (International Light, Newbury, USA). Irradiation was done at 25 °C. Prior to irradiation, media were removed and retained, and the fibroblasts were covered with Ca²⁺/Mg²⁺ (0.01% each) enriched phosphate-buffered saline (PBS) as described previously (1). After irradiation the original media were added back to the fibroblasts. Control fibroblasts were treated in the same manner except that they were not irradiated.

*Hemin Treatment*- A stock solution of hemin (1 mM) was prepared in KOH (8 mM) and phosphate buffer (100 mM, pH 7.4). After removal of the media fibroblasts were treated with hemin (4 μM) in PBS for 1 h. After treatment the fibroblasts were washed thoroughly and the...
original media was added back to the fibroblasts.

_Inactivation of Heme Oxygenase._—To inactivate heme oxygenase, fibroblasts were treated with Sn-protoporphyrin IX (Porphyrin Products, Logan, UT). Immediately after UVA irradiation the PBS was removed from the fibroblasts and Sn-protoporphyrin IX (100 μM) in PBS was added. Fibroblasts were kept in the dark during the treatment with Sn-protoporphyrin IX to minimize photooxidation of the protoporphyrin. After 2 h of treatment at 37 °C the Sn-protoporphyrin IX was removed, fibroblasts were washed and the original media was replaced. A stock solution of Sn-protoporphyrin IX (1 mM) was made up in KOH (8 mM) and phosphate buffer (100 mM, pH 7.4).

_Treatment of Fibroblasts._—To bind low molecular weight intracellular iron fibroblasts were treated with desferrioxamine (Desferal) (500 μM) (Ciba Geigy, Basel, Switzerland) in PBS at 37 °C after removal and storage of the media. After incubation for 1.5 h the desferrioxamine was removed and fibroblasts were washed thoroughly with PBS before adding back the original media.

_Heme Oxygenase-1 mRNA._—Total RNA was isolated by the guanidium thiocyanate-phenol-chloroform method 3 h after irradiation (21). RNA (15 μg/well) was electrophoresed in a MOPS/HCHO 1.3% agarose gel (22), transferred onto a GeneScreen nylon membrane (NEN Research Products, Regensburg, Switzerland), and hybridized with the 1000 base pair EcoRI fragment of the human heme oxygenase cDNA clone 2/10 (3). After autoradiography blots were rehybridized with the PstI fragment (1300 base pairs) of rat glyceraldehyde-3-phosphate dehydrogenase cDNA. The glyceraldehyde-3-phosphate dehydrogenase RNA signal was used as an internal control for the loading error between samples.

_Fibroblast Extracts._—Fibroblasts (8 × 10⁵) were washed thoroughly with ice-cold PBS, harvested with a rubber policeman and homogenized with a Potter Elvehjem homogenizer (Bellco, Fetham, United Kingdom) at 4 °C. Cell debris was removed by centrifugation at 5000 × g, and an aliquot of supernatant was retained for ferritin analysis. The remaining supernatant was spun at 15,000 × g to remove mitochondria, and the new supernatant was centrifuged at 105,000 × g for 60 min. The resulting microsomal pellet was resuspended in phosphate buffer (100 mM, pH 7.4). All centrifugation steps were performed at 4 °C. The protein content of extracts was determined using the method of Bradford (23) standardized with bovine serum albumin.

_Heme Oxygenase Determination._—The method of Shibihara et al. (24) was used to determine heme oxygenase activity. Microsomes (100–200 μg of protein) were incubated with hemin (20 μM), bovine serum albumin (0.064%), NADPH (100 μM), and crude biliverdin reductase extract (0.29 mg ml⁻¹) prepared according to the method of Tammela et al. (25). Reactions took place in phosphate buffer (100 mM, pH 7.4), and samples were gently mixed in the dark at 37 °C for 30 min. The reaction was stopped by placing on ice, tubes were centrifuged at 5000 × g for 1 min at 4 °C, and the absorbance of bilirubin at 465 nm was measured against a base-line absorbance at 520 nm (ε₄₅₅ = 40,000 M⁻¹ cm⁻¹; Ref. 26).

_Ferritin Content._—The ferritin assays were performed with a polyclonal enzyme-linked immunosorbent assay kit (Boehringer, Mannheim, Germany). Supernatants (10–20 μg of protein) from the 5000 × g centrifugation step after homogenization of the fibroblasts were analyzed for ferritin according to the procedure supplied with the kit.

_Heme Content._—Mitochondrial and microsomal pellets, prepared from fibroblasts as described above, were resuspended in 1 ml of concentrated formic acid (Fluka, Buchs, Switzerland), and the heme content of the solution was measured at 398 nm (27). The concentration of heme was calculated from a standard curve constructed from treatment of cytochrome c with concentrated formic acid.

**RESULTS AND DISCUSSION**

This study shows that treatment of human skin fibroblasts (FEK) with 250 kJ m⁻² of UVA irradiation elevated heme oxygenase activity 4-fold, 14 h after irradiation (Fig. 1). The increase in heme oxygenase activity after UVA irradiation we show here extends previous results from this laboratory that have shown induction of heme oxygenase protein levels and increased rate of heme oxygenase-1 RNA accumulation after UVA irradiation of FEK fibroblasts (3, 6). We now show that the UVA-dependent increase in heme oxygenase activity of FEK fibroblasts is paralleled by an increase in ferritin levels. Levels of ferritin were increased approximately 2-fold, 22 h after UVA irradiation and were still maximal 46 h after irradiation (Table 1). Heme oxygenase activity had returned to control levels 46 h after irradiation (Fig. 1). The UVA dose used (250 kJ m⁻²) was equivalent to less than 30 min of exposure to a typical tanning lamp. Thus the UVA-dependent increase in heme oxygenase activity and ferritin levels we show here represent a response to a physiological level of oxidative stress.

Heme oxygenase-1 mRNA levels were increased 13 ± 5-fold (mean ± S.D. of 10 determinations) following irradiation of fibroblasts with 250 kJ m⁻² of UVA 3 days after seeding (Fig. 2). Fibroblasts irradiated 7 days after seeding (100% confluent) showed an even greater increase in heme oxygenase-1 mRNA (Fig. 2). Previous work from this laboratory has shown accumulation of heme oxygenase-1 mRNA in fibroblasts irradiated 2–4 days after seeding (50–500% confluent) (7). For all investigations in the present study fibroblasts 7 days after seeding because they were not undergoing so rapid a rate of growth as that seen in 3-day fibroblasts (data not shown) and therefore more closely represent the growth characteristics of cells in vivo. The apparent discrep-
The induced heme oxygenase activity and a decrease in cytochrome P450 (26). Other studies have shown that iron added as a low molecular weight salt can enhance the rate of ferritin mRNA translation in a variety of cells (20, 36, 37). Iron salts can also act to enhance the rate of ferritin RNA synthesis. However the effect of this enhancement on ferritin protein levels is modest compared to the effect of free iron on translation (36). It has also been proposed that the iron needs to be associated with porphyrin in order to activate ferritin mRNA translation (38). However our results support the alternative proposal that the principal effect of heme on ferritin levels is via free iron (20, 36).

The possibility that UVA irradiation was stimulating ferritin levels by a heme oxygenase-independent mechanism was tested by treating UVA-irradiated fibroblasts with Sn-protoporphyrin IX. This porphyrin irreversibly inhibits heme oxygenase. Sn-protoporphyrin IX treatment of fibroblasts inhibited heme oxygenase activity almost completely (Table I). Under these conditions UVA irradiation had no effect on ferritin levels. This rules out the possibility that iron, released directly from hemes or ferritin by UVA irradiation (39), was stimulating ferritin synthesis. Thus it can be concluded that the effect of UVA on ferritin levels is via heme oxygenase activity and ferritin levels. Treatment of fibroblasts with PBS for the same length of time as fibroblasts were treated with Sn-protoporphyrin IX (2 h) did not effect the UVA-dependent increase in heme oxygenase activity and ferritin levels. Treatment of non-irradiated fibroblasts with Sn-protoporphyrin IX for 2 h inhibited heme oxygenase activity but did not affect basal ferritin levels. To demonstrate that the relationship between heme oxygenase and ferritin was not a specific effect of oxidative stress, we used hemin instead of UVA irradiation as an inducer of heme oxygenase. Fibroblasts treated with hemin showed an increase in heme oxygenase activity of 6-fold 14 h after treatment (from 0.11 ± 0.03 to 0.68 ± 0.07 nmol min⁻¹ mg protein⁻¹, mean ± S.D. of two determinations) and showed an almost 2-fold increase in ferritin 22 h after treatment (from 102 ± 10 to 191 ± 14 ng mg protein⁻¹, mean ± S.D. range of two determinations).

This study has demonstrated that the inducibility of heme oxygenase by oxidative stress plays an important role in the regulation of the major intracellular iron-binding protein, ferritin. It has been hypothesized that an increase in heme oxygenase activity may lead to an increase in the antioxidant potential of cells, thereby enhancing cell survival under oxidative stress (34, 40).
dependent increase in antioxidant potential may actually be mediated by ferritin. We propose that the increased levels of ferritin that result from the UVA-dependent induction of heme oxygenase will further decrease intracellular free iron levels and therefore may limit iron-catalyzed oxidative reactions that would occur during subsequent periods of oxidative stress.

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Addendum—The antioxidant role of ferritin is supported by a study that appeared after submission of this work, showing that the cytotoxicity of hydrogen peroxide and hemin was inhibited by an increase in cellular ferritin levels (41). Balla et al. (41) induced ferritin levels by pre-exposing porcine aortic endothelial cells to hemin. Although they observed an increase in heme oxygenase activity the induction of ferritin was not effected by an inhibitor of heme oxygenase, Sn-mesoporphyrin IX. This result suggests that the induction of ferritin in the endothelial cell system occurs by a mechanism different from the heme oxygenase-dependent pathway we now report to occur in cultured human skin fibroblasts.

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