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Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite

(oxidant stress/inducible response/sunlight)

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ABSTRACT **We have shown that UVA (320–380 nm) radiation, hydrogen peroxide, and sodium arsenite induce a stress protein of ≈32 kDa in human skin fibroblasts. The synthesis and cloning of cDNA from arsenite-induced mRNA populations have now allowed us to unequivocally identify the 32-kDa protein as heme oxygenase. By mRNA analysis we have shown that the heme oxygenase gene is also induced in cultured human skin fibroblasts by UVA radiation, hydrogen peroxide, cadmium chloride, iodoacetamide, and menadione. The known antioxidant properties of heme catabolites taken together with the observation of a high level of induction of the enzyme in cells from an organ not involved in hemoglobin breakdown strongly supports the proposal that the induction of heme oxygenase may be a general response to oxidant stress and constitutes an important cellular defense mechanism against oxidative damage.**

Mammalian cells respond to a wide variety of adverse conditions, both chemical and physical, by inducing the synthesis of a number of stress proteins. The most widely studied of these responses is that induced by heat shock (for review see refs. 1 and 2). Although certain insults other than heat shock itself induce heat shock proteins—for example, treatment of cells with amino acid analogues (3)—others induce stress proteins unrelated to those inducible by heat shock. Both glucose deprivation and anoxic stress are good examples of the latter (4, 5). We have reported (6) the induction of a 32-kDa stress protein in human skin fibroblasts by both near UV radiation (UVA) and the oxidizing agent hydrogen peroxide. Neither of these treatments induced the major heat shock proteins. In addition, we were unable to induce the 32-kDa protein by heat shock in these cells, indicating that the mechanism of induction of this protein is different from that involved in heat shock. Interestingly, a protein of 30–35 kDa had been reported (7–10) as inducible by certain chemical treatments, including heavy metal salts and the sulfhydryl reagent sodium arsenite in rodent, avian, and human cells. In all of these cases the heat shock proteins were also induced. A comparison of the 32-kDa proteins induced by UVA, hydrogen peroxide, and sodium arsenite in human skin fibroblasts by partial peptide mapping indicated that they were identical (6).

We now describe the isolation of cDNA clones corresponding to the mRNA species encoding this stress protein and identify the inducible gene as coding for heme oxygenase. We speculate that this enzyme participates in an inducible protective mechanism against oxidative stress induced by UVA and hydrogen peroxide in human skin cells.

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MATERIALS AND METHODS

Cell Culture and Treatment with UVA and Chemicals. The normal human skin fibroblast cell line EK4 was derived from a foreskin explant, in this laboratory, and cultured routinely as described (11). Cells were seeded into plastic culture dishes (30–50% confluence) 2–4 days prior to either chemical treatment or UVA irradiation. To treat cells with chemicals, the growth medium was removed and reserved, the cells were rinsed with isotonic phosphate-buffered saline (PBS), and the chemical was added at the indicated concentration in PBS. Treatments were for 30 min at 37°C, unless otherwise indicated. For UVA irradiation, medium was removed and reserved, and cells were rinsed with PBS plus calcium and magnesium and irradiated through this buffer at ambient temperature. UVA radiation was provided by a broad-spectrum (330–450 nm) Uvasun 3000 lamp (Mutzhas, F.R.G.) and dose rates were monitored as described (12). Irradiation times were <15 min and control dishes were sham-irradiated. After treatments the growth medium was added back to the cells and incubation was continued for the times indicated in the figure legends before isolation of total RNA.

Synthesis of cDNA and Library Construction. Total RNA was isolated from EK4 fibroblasts (≈1.4×10^8 cells) 2 hr after treatment with sodium arsenite (50 μM for 30 min) by using the ribonucleoside–vanadyl complex method of Berger and Birkenmeier (13) and poly(A)$^+$ RNA was then isolated from this total RNA by oligo(dT)-cellulose (type 3, Collaborative Research) affinity chromatography (14). The induced poly(A)$^+$ RNA population was then used to prepare cDNA by using the improved reverse-transcription method of Gubler and Hoffman (15). For the preparation of our first library, this cDNA was cloned directly into the λ insertion vector λ ZAP (Stratagene) by using standard techniques (16). In preparing a second library, the cDNA was first size-fractionated by electrophoresis on a 5% polyacrylamide gel in Tris borate buffer and only cDNAs of >1 kilobase (kb) were cloned into the λ ZAP vector.

Screening of cDNA Libraries. The cDNA inserts cloned into the λ ZAP vector can be automatically excised and recircularized into a smaller and more easily manipulated plasmid vector (Bluescript) by using an f1 helper phage (17). We first prepared a sublibrary of these recombinant plasmids from our unfractionated cDNA library by using the helper-phage rescue protocol. These DNAs were then screened by using a modified hybrid-selection protocol (18) and two cDNA clones (clone 1/4, 250 base pairs; clone 1/149, 500 base pairs) were isolated.

After transfer of λ ZAP plaques to nitrocellulose, further screening of our unfractionated cDNA library by using a ^{32}P-labeled DNA probe derived from clone 1/149 failed to identify any positive clones with longer inserts so that a second, size-fractionated library was prepared and screened.

DNA Sequence Analysis. cDNA inserts were sequenced by using a modification of the Sanger dideoxyribonucleotide procedure, which uses strand-specific primers (SK and KS primers, Stratagene) and modified T7 DNA polymerase (Sequenase, United States Biochemical) to sequence directly from the double-stranded plasmid DNA (19).

Partial Peptide Mapping. Partial peptide maps were obtained from proteins in excised gel fragments by using *Staphylococcus aureus* V8 protease exactly as described by Cleveland *et al.* (20).

DNA Probe Preparation and Northern Analysis. ^{32}P-labeled DNA probes for both library screening and Northern analyses were prepared from restriction fragments isolated from low-melting-temperature agarose gels (1%) by random-primed DNA synthesis by the method of Feinberg and Vogelstein (21). Total RNA for Northern blotting was isolated by the one-step acid guanidium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (22). Equal (15 μg) quantities of RNA were loaded into each slot of Mops/formaldehyde agarose (1.3%) gels. After electrophoresis, RNA was capillary-blotted onto GeneScreen (NEN Research Products) and hybridized to ^{32}P-labeled DNA probes by using standard techniques (16). A ^{32}P-labeled probe derived from a cDNA fragment (1400 base pairs, *Pst* I) of the rat glyceraldehyde phosphate dehydrogenase gene (ref. 23; kindly provided by P. Amstad, Swiss Institute for Experimental Cancer Research) was used as a loading control.

RESULTS

Cloning of the Sodium Arsenite-Inducible Gene. To confirm that the apparent induction of a 32-kDa protein in human skin fibroblasts was due to stress-regulated control of gene expression, we isolated poly(A)$^+$ RNA from cells 2 hr after treatment with sodium arsenite and compared the *in vitro* translation products with those originating from control mRNA. The results in Fig. 1*A* show clearly that only mRNA from the treated cells gives rise to high levels of a 32-kDa protein and that this product constitutes 2–3% of the total protein synthesized. The high levels of the 32-kDa protein encouraged us to initiate cDNA synthesis and library construction by using the induced mRNA population with no further enrichment. With a sublibrary of recombinant Bluescript plasmids and the hybrid selection technique, we were able to identify two pools of five clones among 16 pools tested whose DNA hybridized to an mRNA species that gave rise to a 32-kDa translation product. Two clones, designated clones 1/4 and 1/149, with insert sizes of ≈250 and 500 base pairs, respectively, were isolated from these pools by a second round of hybrid selection (Fig. 1*B*).

To determine whether the cDNAs isolated corresponded to an inducible mRNA in sodium arsenite-treated fibroblasts, we used Northern RNA gel blot analysis (Fig. 2). This experiment clearly demonstrated that a ^{32}P-labeled probe derived from the insert of clone 1/149 hybridizes to an mRNA species of ≈1.6 kb and that this species is strongly inducible by sodium arsenite treatment. Finally, as additional proof that we have cloned a fragment of the gene that corresponds to the 32-kDa stress protein induced *in vivo* by sodium arsenite, we isolated the stress protein from excised NaDodSO$_4$/polyacrylamide gel fragments from the following three sources: (*i*) total protein from arsenite-treated fibroblasts, (*ii*) total protein translated *in vitro* from poly(A)$^+$ RNA isolated from arsenite-treated cells, and (*iii*) protein translated *in vitro* from mRNA that had been hybrid-selected by clone 1/149 DNA. Analysis by peptide mapping clearly showed that the proteins isolated from the three sources are identical (Fig. 3).

Subsequent screening of a second cDNA library prepared in λ ZAP by using size-selected (>1 kb) cDNAs led to the isolation of a recombinant (designated clone 2/10) that had an insert of ≈1.5 kb. Partial DNA sequence analysis of clones

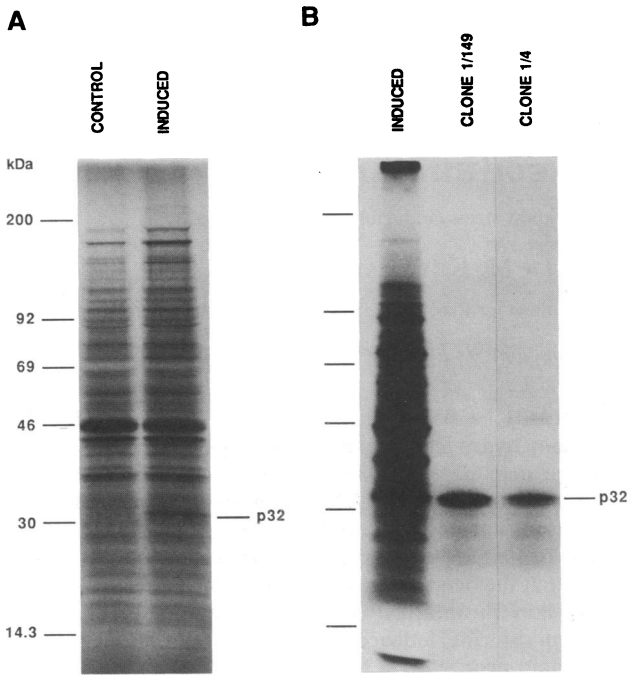

FIG. 1. (*A*) Autoradiogram showing an NaDodSO$_4$/PAGE analysis of ^{35}S-labeled *in vitro* translation products of poly(A)$^+$ RNA isolated from cells 2 hr after treatment with sodium arsenite (induced) or from untreated cells (control). (*B*) Fluorogram showing an NaDodSO$_4$/PAGE analysis of ^{35}S-labeled *in vitro* translation products of poly(A)$^+$ RNA hybrid-selected by cDNA clones 1/149 and 1/4. For reference the pattern obtained from total poly(A)$^+$ RNA isolated from sodium arsenite-treated (induced) cells is shown. Molecular mass markers are indicated to the left in this and subsequent figures. The position of the inducible 32-kDa protein (p32) is also indicated.

1/4, 1/149, and 2/10 revealed a 3′ sequence and 364 nucleotides derived from the putative 5′ end of the cDNA (Fig. 4). A computer search (EMBL data base, Release January 14, 1988) revealed that our 5′ sequence showed high homology with the cDNA corresponding to the gene coding for rat heme oxygenase. Further comparison with the more recently published sequence for human heme oxygenase cDNA (24) revealed essentially 100% homology at the nucleotide level over both the partial 5′ coding sequence and the 3′ untranslated region of the mRNA (Fig. 4); a clear demonstration that

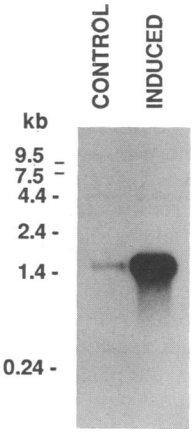

FIG. 2. Northern hybridization of a ^{32}P-labeled DNA probe derived from the 500-base-pair cDNA insert of clone 1/149 with poly(A)$^+$ RNA (3 μg per lane) derived from control or sodium arsenite-treated (induced) human skin fibroblasts.

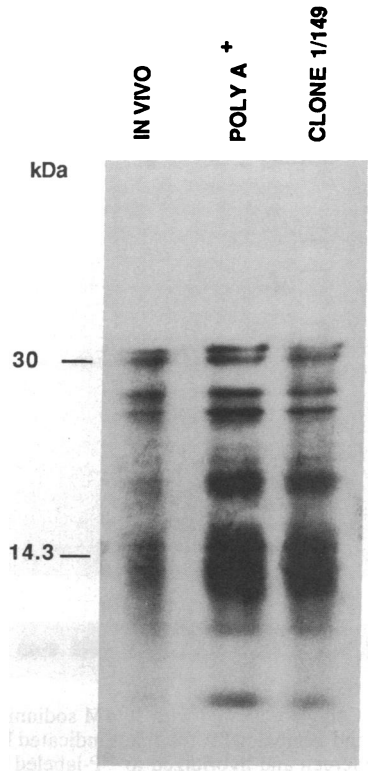

FIG. 3. Comparison of 32-kDa stress proteins by partial proteolytic digestion using *Staphylococcus aureus* V8 protease. The stress protein was isolated from cells labeled with ^{35}S after treatment with sodium arsenite (*in vivo*), from the *in vitro* translation products of poly(A)$^+$ RNA isolated from arsenite-treated cells (polyA$^+$), and from the *in vitro* translation of poly(A)$^+$ RNA hybrid-selected by cDNA clone 1/149 (clone 1/149) and compared by using the method of Cleveland *et al.* (20).

the sodium arsenite-inducible gene codes for the enzyme heme oxygenase.

Accumulation of Heme Oxygenase mRNA After Stress Treatments. After treatment of human skin fibroblasts with 50 μM sodium arsenite, the levels of heme oxygenase mRNA increased above the control level within 1 hr of treatment with a maximum level of induction seen between 2 and 4 hr after treatment (Fig. 5*A*). Elevated levels of heme oxygenase mRNA persist for 6 hr and then decline, but only after 24 hr of incubation did the levels of heme oxygenase mRNA approach the low levels seen in untreated cells. Heme oxygenase mRNA was also induced by treatment with either a broad spectrum UVA source (Fig. 5*B*) or hydrogen peroxide (Fig. 5*C*). The kinetics of mRNA accumulation are similar to those seen after treatment with sodium arsenite but the maximum levels of induction are somewhat lower.

The influence of a range of different stress conditions on the levels of heme oxygenase mRNA is shown in Fig. 6. In contrast to a recent study in which somewhat different heat shock conditions were employed (24), we observe that treatment of human cell populations for 5 min at 45°C did lead to a slight (2-fold, by densitometry) increase in heme oxygenase mRNA levels. Both menadione, which has been shown to induce high levels of the 32-kDa stress protein, and iodoacetamide (a thiol-reactive compound) were potent inducers of the specific mRNA. However, the best inducer under our experimental conditions was the metal salt, cadmium chloride (Fig. 6).

DISCUSSION

A stress protein, whose molecular mass ranges from 30 to 35 kDa, is induced in avian, rodent, and human cells by a variety

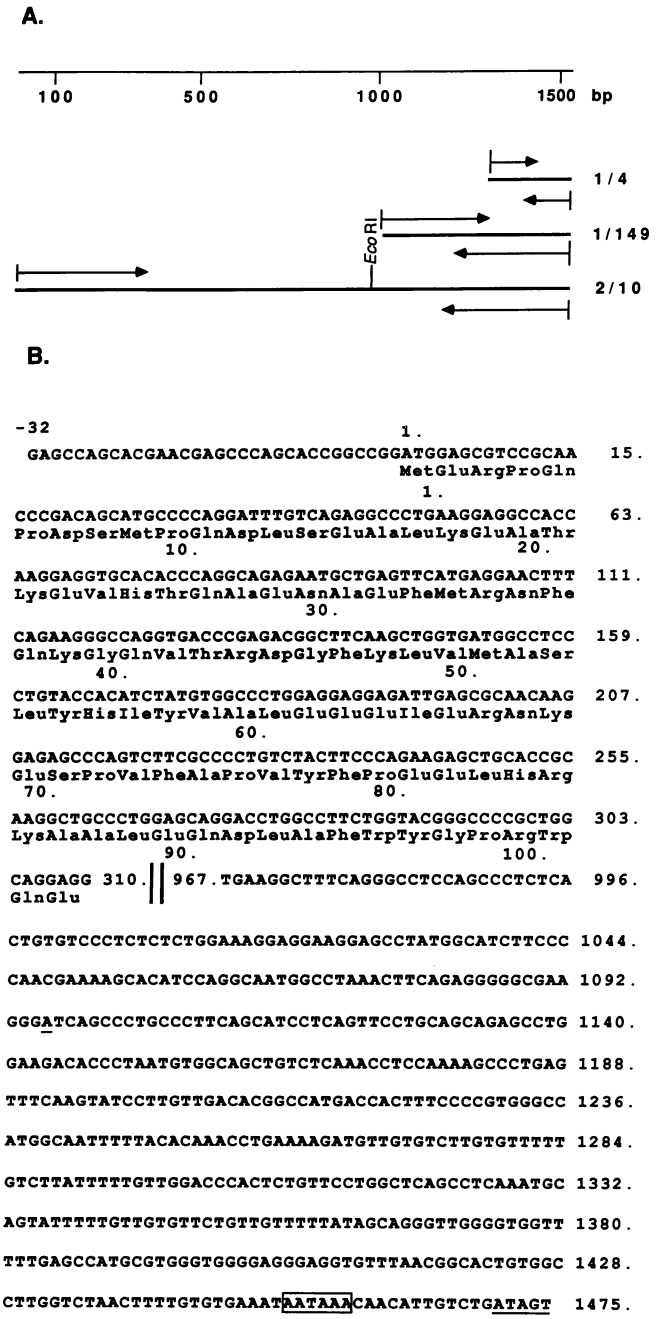

FIG. 4. (*A*) DNA sequence relationship among clones 1/4, 1/149, and 2/10. Arrows indicate both the direction and extent of sequence determinations. (*B*) Comparison of the nucleotide sequences derived from clones 1/4, 1/149, and 2/10 with the cDNA sequence of human heme oxygenase. Nucleotide numbering is based on the full cDNA sequence determined by Yoshida *et al.* (24). Only the nucleotide sequence determined from clones 1/4, 1/149, and 2/10 is shown and reproducible differences from the published sequence are underlined. The putative polyadenylylation signal is boxed.

of chemical treatments (7–10). The common link between all of these studies with cells derived from different species is that sodium arsenite is always an efficient inducer of these stress proteins in the different cell systems employed. By preparing a cDNA library from mRNA extracted from sodium arsenite-treated human skin fibroblasts and selecting for clones that hybridize to inducible mRNAs that can be translated *in vitro* into 32-kDa proteins, we have isolated exclusively cDNA clones whose DNA sequence (Fig. 4*B*) corresponds to the human gene coding for the enzyme heme oxygenase (32.8 kDa). By molecular analysis with this cloned

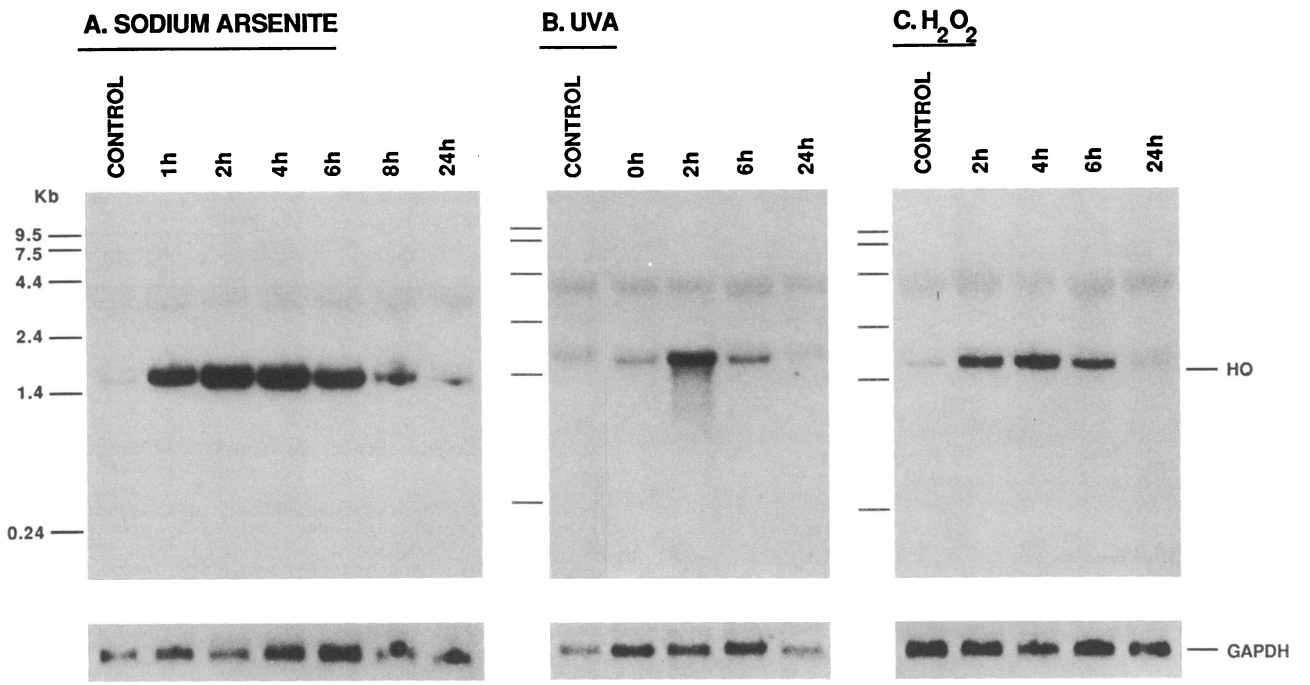

FIG. 5. Kinetics of heme oxygenase mRNA accumulation in human skin fibroblasts. Cell were treated with 50 μM sodium arsenite for 30 min (*A*), UVA radiation at 2×10^5 J/m^2 (*B*), or 100 μM hydrogen peroxide for 30 min (*C*) and incubated for the times indicated before isolation of total RNA. After Mops/formaldehyde electrophoresis, RNA was transferred to GeneScreen and hybridized to ^{32}P-labeled probes derived from the 1-kb *Eco*RI fragment of clone 2/10 and, as a loading control, the *Pst* I fragment of the glyceraldehyde phosphate dehydrogenase gene.

cDNA, we have shown unequivocally that the 32-kDa stress protein induced by sodium arsenite is heme oxygenase and that this enzyme is also the major stress protein induced in human skin cells exposed to near UV radiation (UVA) and the oxidizing agent hydrogen peroxide. A unique inducible mRNA species that hybridizes to our cloned cDNA is also observed after treatment with a second sulfhydryl reagent, iodoacetamide, a heavy metal salt, cadmium chloride, and a redox cycling quinone, menadione (Fig. 6). Therefore, our results leave little doubt that the 32-kDa stress protein previously shown to be inducible by sulfhydryl reagents and a variety of heavy metal salts is also heme oxygenase. Indeed, since the submission of this report, a study has been published (25) that provides evidence that the 32-kDa protein induced in mouse fibroblasts by a variety of agents including heat shock, metal salts, and tumor promoters is a mouse homologue of heme oxygenase. The results of this and the present study are compatible with earlier observations in which heme oxygenase enzyme activity was shown to be increased in the livers of rats treated with many agents including heavy metal salts, such as cadmium chloride and lead acetate, and sulfhydryl reactive compounds, such as diethyl maleate (26, 27). Two genes code for two isozymes of rat heme oxygenase (HO1 and HO2), only one of which (HO1) is inducible by hematin and other chemical agents (28). Heme oxygenase is inducible by heat shock in rats (29) and the data in Fig. 6 indicate that it may also be a minor heat shock protein in humans (but see refs. 6 and 24).

Heme oxygenase plays a defined and essential role in heme catabolism by cleaving heme to form biliverdin (30). Biliverdin is subsequently converted to bilirubin by biliverdin reductase. It has been suggested that heme oxygenase plays a major role in the regulation of biotransformation reactions that depend on cytochrome P450 (28). In addition, there may be considerable advantages in rapidly mobilizing this enzyme in response to certain stress conditions even in tissues, such as the skin, that are not normally major sites of heme catabolism. Two specific inducers of heme oxygenase mRNA in human skin cells are UVA irradiation and treatment with

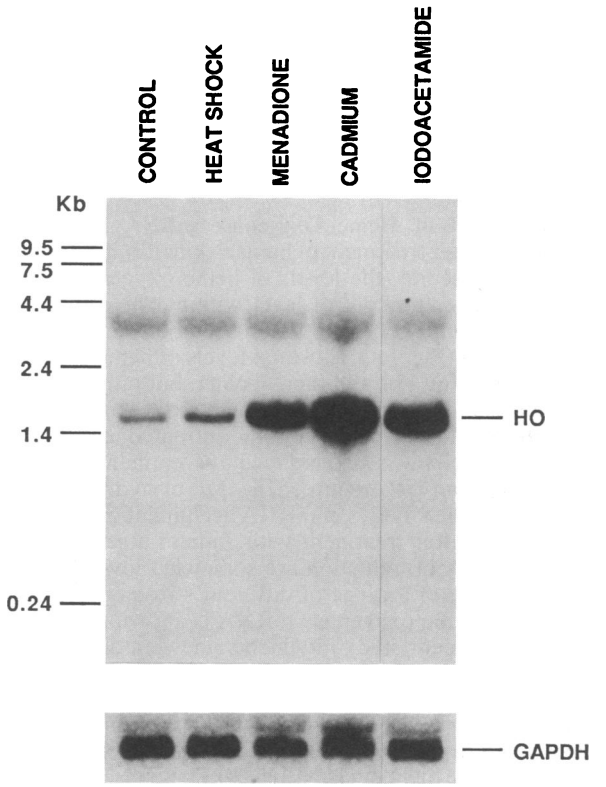

FIG. 6. Effects of chemical treatments and heat shock on heme oxygenase mRNA levels in human skin fibroblasts. Cells were treated as follows: heat shock for 5 min at 45°C, 500 μM menadione for 30 min, 100 μM cadmium chloride for 3 hr, and 50 μM iodoacetamide for 30 min. With the exception of the cadmium chloride-treated cells, from which total RNA was extracted immediately after treatment, cells were incubated for 3 hr before isolation of total RNA. Northern analysis was exactly as described in Fig. 5.

hydrogen peroxide (Fig. 5 *B* and *C*), both of which result in a condition of cellular oxidant stress and have been shown to induce the stress protein (6). An increase in heme oxygenase activity will increase cellular capacity to generate both biliverdin and bilirubin providing that biliverdin reductase is present. Unconjugated bilirubin is an efficient scavenger of singlet oxygen (31) and is able to react with superoxide anion and peroxyl radicals (32, 33). Stocker *et al.* (34) have studied the antioxidant properties of bilirubin bound to albumin (as it would be in the blood) and found that, as a result of its scavenging activity toward oxygen radicals, it is able to prevent oxidative damage both to albumin itself and, by preventing lipid peroxidation, to albumin-bound fatty acids. On the basis of these studies, they have suggested that bilirubin may be a physiological antioxidant in plasma and the extravascular space and have noted the apparent relationship between heme oxygenase induction and oxidant stress.

Since many of the potential cellular forms of the products of heme catabolism react efficiently with peroxyl radicals (33, 35), these products may also play a direct role in cellular defense against oxidant damage. The remarkable level of induction of heme oxygenase mRNA in cultured skin cells provides strong support for such a mechanism and suggests that the protective pathway is specifically activated under conditions of oxidant stress. The destruction of endogenous heme compounds may protect cells specifically against UVA stress by an additional pathway. UVA irradiation of heme-containing proteins can generate potentially lethal damage by singlet oxygen generation in bacterial cells (36). A temporary reduction in heme proteins could constitute a protective response against such radiation damage in human skin cells.

The potential antioxidant defense provided by the induction of heme oxygenase adds to the powerful constitutive defense provided by endogenous glutathione against the cytotoxic consequences of both UVA/UVB radiation (37, 38) and hydrogen peroxide (unpublished data). Furthermore, all the agents that induce heme oxygenase that we have described here also interact with and probably reduce the levels of available glutathione. Thus the signal for induction of the enzyme may be related to modified or reduced levels of this ubiquitous nonprotein thiol.

In conclusion, our results identify heme oxygenase as the 32-kDa stress protein induced in human cells by a variety of stress treatments including UVA, oxidizing agents, and heavy metal salts. Induction of this enzyme could function as part of an inducible protective response against oxidative damage.

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