[CANCER RESEARCH 51, 974–978, February 1, 1991]

Induction of Heme Oxygenase: A General Response to Oxidant Stress in Cultured Mammalian Cells[1]

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ABSTRACT

Accumulation of heme oxygenase mRNA is strongly stimulated by treatment of cultured human skin fibroblasts with ultraviolet radiation, hydrogen peroxide, or the sulfhydryl reagent sodium arsenite (S. M. Keyse and R. M. Tyrrell. Proc. Natl. Acad. Sci. USA, *86:* 99–103, 1989). Since this will result in a transient reduction in the prooxidant state of cells, the phenomenon may represent an important inducible antioxidant defense mechanism. To examine the generality of the response, we have measured the accumulation of the specific mRNA in a variety of human and mammalian cell types after inducing treatments. Induction by sodium arsenite is observed in all additional human cell types tested. This includes primary epidermal keratinocytes and lung and colon fibroblasts as well as established cell lines such as HeLa, TK6 lymphoblastoid, and transformed fetal keratinocytes. Strong induction of heme oxygenase mRNA is also observed following sodium arsenite treatment of cell lines of rat, hamster, mouse, monkey, and marsupial origin. The agents which lead to induction in cultured human skin fibroblasts fall into two categories: (*a*) those which are oxidants or can generate active intermediates (ultraviolet A radiation, hydrogen peroxide, menadione, and the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate); (*b*) agents which are known to interact with or modify cellular glutathione levels (buthionine sulfoximine, sodium arsenite, iodoacetamide, diamide, and cadmium chloride). These observations strongly support the hypothesis that induction of the enzyme is a general response to oxidant stress in mammalian cells and are consistent with the possibility that the cellular redox state plays a key role.

INTRODUCTION

Constitutive cellular protection against oxidation is provided by various cellular compounds such as glutathione (1, 2) and antioxidant enzymes that include catalase, superoxide dismutase, and glutathione peroxidase. Although a regulon involved in the induction of several antioxidant enzymes has been characterized in bacteria (3), these enzymes are, at most, only slightly inducible by oxidants in eukaryotic cells (*e.g.*, Refs. 4 and 5). In view of the increasing evidence for the involvement of cellular oxidation in various pathological processes including cancer formation (6), it is of considerable interest to determine whether mammalian cells possess inducible pathways for antioxidant defense. We have recently proposed (7, 8) that the transient induction of high levels of heme oxygenase gene expression provides such a pathway since enhanced enzyme activity will lead to a reduction in the potential prooxidant catalytic activity provided by heme and associated compounds.

Heme oxygenase is induced in whole animal tissues (particularly kidney and liver) by a series of agents which include heme compounds, heavy metals, and hormones (for reviews see Refs. 9 and 10). The isolation of cDNA[3] and genomic clones for both the rat and human heme oxygenase genes (7, 11–14) should facilitate studies aimed at elucidation of the protein factors and genetic elements involved in the inducible regulation of gene expression. In parallel with these studies there have been various reports of induction of low molecular weight stress proteins by such diverse agents as heavy metals (15–17), sulfhydryl reagents (16, 18, 19), and the tumor promoter TPA (17) as well as UVA (320–380 nm) radiation and oxidants (18). Subsequent studies have provided a reasonable indication that the protein induced was, in all cases, heme oxygenase (7, 20).

In the present study we further investigate the generality of this response in cells derived from a variety of human tissues and mammalian species. In particular, we focus on the possibility that heme oxygenase, the primary function of which is to catabolize hemoglobin, may have an important secondary role in antioxidant defense.

MATERIALS AND METHODS

Cell Strains and Culture. The normal human fibroblast cell line FEK_4 and the keratinocyte cell line EK_4 were derived from a foreskin explant in this laboratory and cultured as described previously (21). Fibroblasts (FEK_4) were routinely cultured in Earle's modified minimal essential medium supplemented with 15% FCS, glutamine, sodium bicarbonate, penicillin, and streptomycin. Cells were passaged every 5–8 days by trypsinization and were used for experiments between passages 6 and 15. Human lung fibroblasts (WI-38; American Type Culture Collection), human colon fibroblasts (established from a tissue explant kindly provided by Dr. H. Tevaearai), and HeLa cells (kindly provided by Dr. Peter Beard) were maintained as described above for the FEK_4 cells, except that HeLa cells were maintained in 10% FCS and split every 3 days. The transformed fetal keratinocyte cell line [HFK-SV61 (22)] was also grown in the same medium as the primary fibroblasts, except that only 10% FCS was added, and the medium was supplemented with 40 μg/ml of hydrocortisone. These cells were passaged twice a week at a 1:2 or 1:4 ratio in order to maintain healthy growth.

In addition to the human lymphoblastoid cell line, TK6 (23), four lymphoblastoid cell lines were used which had been deliberately transformed by Epstein-Barr virus. Two were derived from BCNS patients (L3 and L4) and two were derived from corresponding age-matched controls (L1 and L2). All lymphoblastoid cell populations were grown in suspension in RPMI 1640 supplemented with 10% FCS, glutamine, sodium bicarbonate, penicillin, and streptomycin. Cells were maintained in exponential growth by diluting to 2×10^5 cells/ml every 2–3 days.

Mouse fibroblast (Swiss 3T3; Flow), mouse epidermal (JB6, kindly provided by Dr. Paul Amstad), rat kidney fibroblast, Chinese hamster ovary, African green monkey kidney (CV-1), and *Monodelphis domestica* fibroblast (MODO) cells were cultured as described above for human fibroblast cell lines and split when necessary.

UV Sources and Irradiation Conditions. Cells which grew in monolayer were plated (1×10^6 cells/dish) in 20-cm-diameter Falcon tissue

Received 9/13/90; accepted 11/20/90.

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[1] The work reported in this paper was supported by grants from the Swiss National Science Foundation (3.186.088) and the Swiss League Against Cancer and was undertaken during the tenure of a Research Training Fellowship to L. A. A. by the International Agency for Research on Cancer.

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[3] The abbreviations used are: cDNA, complementary DNA; TPA, tetradecanoylphorbolacetate; UVA, ultraviolet A; FCS, fetal calf serum; BCNS, basal cell nevus-syndrome; PBS, phosphate-buffered saline; SA, sodium arsenite; SSC, standard saline-citrate (0.15 M NaCl-0.015 M trisodium citrate 2-hydrate-0.1% sodium dodecyl sulfate); HO, heme oxygenase.

culture dishes and grown to 75% confluency. Just prior to irradiation of cells, the growth medium was removed and reserved, and the monolayer was rinsed twice with PBS and then covered with buffer (10 ml). UV dose rates were monitored as described previously (21). Lymphoblastoid cells were collected in rapid growth phase by centrifugation and washed twice in PBS. For UVA treatments, cell populations were irradiated using a Uvasun 3000 lamp (Mutzhas, Munich, Germany) which emits wavelengths between 330 and 450 nm at a dose rate of 300 W/m^2 at the standard irradiation position. UVC (254 nm) radiation at a dose rate of 2.0 W/m^2 was obtained from a germicidal lamp. UVB radiation was provided by a bank of Philips TL 40W/01 experimental lamps (a generous gift from Philips) at a dose rate of 11.0 W/m^2. These lamps emit approximately 90% of the energy in the UVB (290–320 nm) region between 310 and 315 nm and approximately 10% in the UVA region. Ionizing radiation was obtained from a ^{137}Cs gamma source. After irradiation, reserve medium was replaced and cell populations were incubated for various periods of time prior to RNA extraction. Survival of colony-forming ability was measured as described previously (21).

Chemical Treatments. For chemical treatment, monolayer cultures were washed with PBS as described above and the appropriate chemical was added to the PBS: (*a*) SA, 50 μM for 30 min; (*b*) hydrogen peroxide, 100 μM for 30 min; or (*c*) TPA, 20 ng/ml for 30 min. In the case of lymphoblastoid populations, cells were centrifuged, washed, and treated using approximately 5 × 10^6 cells/sample. After the treatment period, cells were washed twice and the reserve medium was replaced for incubation.

Analysis of mRNA. Total RNA was prepared by the one-step acid guanidinium thiocyanate/phenol/chloroform method (24). Cells were lysed in 4 M guanidinium thiocyanate solution (0.5% sodium *N*-lauryl-sarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M β-mercaptoethanol) and the extraction procedure was carried out on ice. RNA was quantitated by absorption spectrophotometry and 2 μg of total RNA from each sample were electrophoresed on 1% Tris-borate-EDTA agarose gels to control for quality. Total RNA (15 μg/sample) was then electrophoresed on 3-(*N*-morpholino)propanesulfonic acid-formaldehyde agarose (1.3%) gels. After electrophoresis, RNA was capillary blotted onto GeneScreen (NEN Research Products, Boston, MA). Membranes were baked, prehybridized, and hybridized at 42°C to ^{32}P-labeled probes prepared by using random primed DNA synthesis of restriction fragments of the cDNA of human heme oxygenase [clone 2/10 (7)]. The filters were washed three times at room temperature with a solution containing 2 × SSC and then washed once at room temperature in 0.1 × SSC and twice at 42°C with 0.1 × SSC. Graded increases in temperature were then used to decrease background when necessary. The filter was exposed to preflashed Amersham hypersensitive film and the autoradiograph obtained was scanned by densitometry.

After stripping, filters were rehybridized with a ^{32}P-labeled probe derived from a 1400-base pair cDNA fragment of the rat glyceraldehyde phosphate dehydrogenase gene (a kind gift of P. Amstad) as a loading control.

RESULTS

Stimulation of Heme Oxygenase mRNA Expression in Cultured Human Skin Fibroblasts by Treatment with Various Agents. We have previously observed heme oxygenase mRNA induction after treatment of cultured human skin fibroblasts with UVA radiation, H_2O_2, and SA (7). We now compare the kinetics and levels of accumulation of the mRNA observed after treatment with these agents with that obtained by treatment with the tumor promoter, TPA, which has been shown to induce the protein (17) and the mRNA (20) in cultured mouse cells. Total RNA was isolated at various times after induction for up to 24 h and electrophoresed on 3-(*N*-morpholino) propanesulfonic acid-formaldehyde gels in order to prepare Northern blots for heme oxygenase mRNA analysis. As shown in Fig. 1, the accumulation of mRNA reached maximum levels within 2–4 h after treatment for all 4 agents. The approximately 13-fold induction over basal levels observed after TPA treatment (20 ng/ml for 30 min) was similar to that observed for a 30-min treatment with either 50 μM SA or 100 μM H_2O_2. In all cases, levels of mRNA accumulation dropped to basal values within 8 h after treatment.

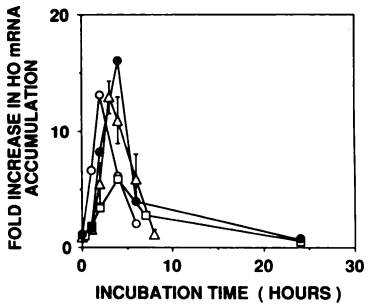

Fig. 1. Fold increase in HO mRNA accumulation induced in human fibroblast populations as a function of posttreatment incubation time. Treatments were with SA (●) (50 μM, 30 min), H_2O_2 (△) (100 μM, 30 min), TPA (○) (20 ng/ml, 30 min), or UVA (□) (single dose of 0.2 MJ/m^2). Except for the results with H_2O_2, for which the mean of several determinations is represented, the results shown are for a single representative experiment.

Table 1 *Induction of heme oxygenase mRNA accumulation in human skin fibroblasts*

Treatment	Fold increase in mRNA at 3 h posttreatment
Ionizing radiation (rads)	
10	2.1 ± 0.3
20	2.6 ± 0.2
50	3.2 ± 0.5
100	2.9 ± 0.6
200	3.1 ± 0.5
400	3.2 ± 0.4
800	3.7 ± 0.5
1000	4.0 ± 0.3
UVC (J/m^2)	
5	0
10	2.3 ± 0.5
20	2.4 ± 0.1
50	2.4 ± 0.3
UVB (J/m^2 × 10^{-3})	
5	0
10	1.5 ± 0.2
20	2.5 ± 0.1
50	2.9 ± 0.1

In addition to the above agents, cell populations were treated with 3 types of radiation (UVC, UVB, and ionizing) known to cause high levels of damage to DNA. The maximum doses used were chosen so as to allow survival of a significant fraction of the cell populations. Levels of mRNA accumulation were measured in samples extracted after 3 h of incubation following treatment (Table 1). All three types of radiation increased basal mRNA levels by a maximum of only 3–4-fold. Furthermore, close to maximum levels were reached at the low end of the dose range in all cases. A similar dose-response relationship was also determined for UVA radiation (see also Ref. 8) and data for mRNA accumulation by all three UV wavelength ranges is shown plotted as a function of surviving fraction (Fig. 2). The dramatic increase in HO mRNA accumulation is clearly a characteristic of the UVA region and the small induction by the shorter wavelengths is probably the result of a less specific pathway stimulated by cell destruction.

Induction of Heme Oxygenase mRNA Accumulation in Human Cells Cultured from Different Tissues. The target cells for our initial studies that demonstrated the induction of heme oxygenase mRNA accumulation by UVA radiation and other forms of

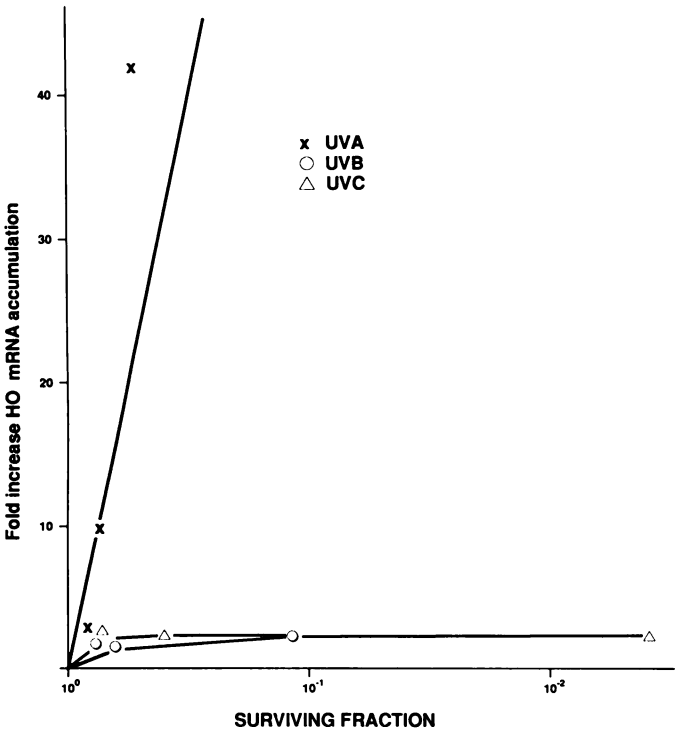

Fig. 2. Fold increase in HO mRNA accumulation as a function of the fraction of fibroblast cell populations surviving treatment over a range of doses of UVA (×), UVB (○), and UVC (△) radiations.

oxidant stress were fibroblasts cultured from human foreskin (7). To determine the generality of the response in human tissues, we have selected standard conditions for sodium arsenite treatment and followed the kinetics of induction in a variety of primary and transformed human cell lines (Fig. 3). Induction was observed in all cell types tested and maximum values of mRNA accumulation were observed 2–4 h after treatment. In primary fibroblast cell lines derived from skin, lung, and colon (Fig. 3, *A* and *B*), maximum induction levels reached 15–20-fold over basal levels whereas less than 10-fold induction was seen in primary epidermal keratinocytes after similar treatment (Fig. 3*A*). Permanent cell lines such as HeLa (Fig. 3*C*) or fetal keratinocytes transformed with origin-defective SV40 (Fig. 3*D*) show moderate induction in the range 10–15-fold. The largest induction seen under these treatment conditions was in the human lymphoblastoid line (TK6; Fig. 3*E*) which displayed a larger than 30-fold increase in HO mRNA levels.

To examine further the induction in lymphoblastoid cell lines, we have measured mRNA accumulation in a series of Epstein-Barr virus-transformed lymphocyte cell lines after treatment with sodium arsenite, hydrogen peroxide, and UVA radiation (Table 2). Two of these cell lines were derived from BCNS patients since fibroblast cell lines derived from such patients have been reported to show enhanced susceptibility to long wavelength UV (25). The fold increases in mRNA accumulation vary over a wide range (5–28) but despite reproducibility between experiments using the same agent and cell line, the differences appear to reflect a large range of variation between individuals rather than differences between normal and BCNS individuals.

Induction of Human Heme Oxygenase mRNA Accumulation in Cells Cultured from Different Mammalian Species. In addition to induction of expression of the heme oxygenase gene in human cells (7, 14), expression of the gene is also inducible in

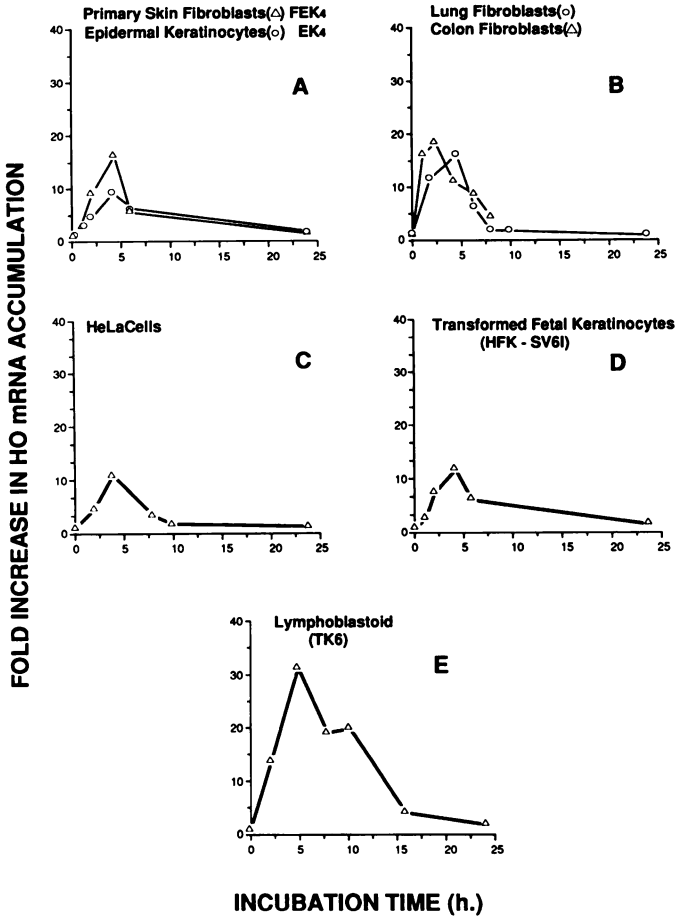

Fig. 3. Fold increase in HO mRNA accumulation at various incubation times following treatment with sodium arsenite (50 μM, 30 min) in a range of primary and transformed cell types derived from different human tissues. Results shown are for a single representative experiment.

Table 2 *Induction of heme oxygenase mRNA accumulation in human lymphoblastoid cell lines*

Lymphoblastoid[a] cell line	Inducing agent[b]		
	SA	H_2O_2	UVA
TK6	31.2[c]	8.4	8.0
L1	12.4	5.5	14.2
L2	28.1	12.0	24.0
L3	25.8	13.3	18.7
L4	22.8	12.3	9.4

[a] L1 and L2 are Epstein-Barr virus-transformed lymphocytes from control individuals which have been age- and sex-matched to Epstein-Barr virus-transformed lymphocytes derived from basal cell nevus syndrome patients (L3 and L4, respectively).

[b] Agents used for stimulation of heme oxygenase mRNA accumulation were sodium arsenite (50 μM, 30 min), hydrogen peroxide (100 μM, 30 min), or ultraviolet-A radiation (0.2 M Jm^{-2}).

[c] Peak accumulation of heme oxygenase mRNA shown as fold increase over basal level expression. Peak accumulation occurred between 4 and 8 h of incubation following treatment.

cells cultured from rat (11) and mouse (26) by various agents including the major substrate of the enzyme, heme. To further examine the species distribution of this inducible response, we have examined the stimulation of mRNA accumulation after sodium arsenite treatment of cells cultured from a variety of mammalian species including monkey, mouse, rat, hamster, and opossum (Fig. 4). Accumulation of HO mRNA is stimulated between 7- and 14-fold in cells cultured from all species tested with peak levels occurring between 2 and 4 h posttreatment incubation.

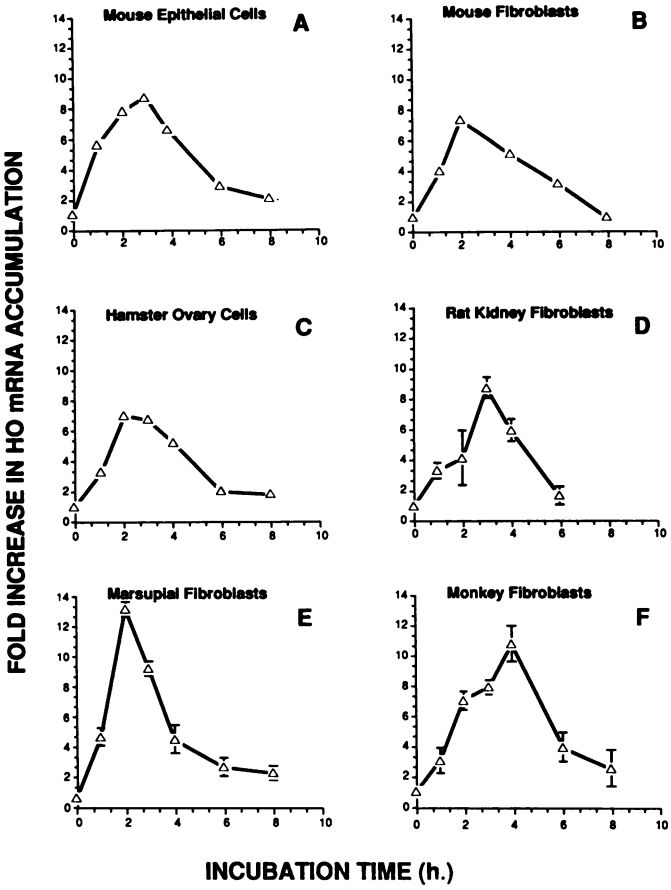

Fig. 4. Fold increase in HO mRNA accumulation at various incubation times following treatment with sodium arsenite (50 μM, 30 min) in a range of cultured cell types derived from a variety of mammalian species. *Bars* are shown only where results represent the mean of more than two independent determinations.

Table 3 *Agents which induce heme oxygenase mRNA in cultured human skin fibroblasts*

Agents which generate active oxygen intermediates	Agents which interact with and/or modify glutathione levels
UVA radiation	Buthionine sulfoximine
Hydrogen peroxide	Sodium arsenite
Menadione	Iodoacetamide
TPA	Cadmium chloride
γ-Radiation	Diamide

DISCUSSION

The data described herein clearly demonstrate that the stimulation of expression of the heme oxygenase gene is a general mammalian stress response that occurs in all cultured human cell types tested (Fig. 3) and in a range of mammalian species from opossum to human (Fig. 4) after treatment of cell populations with a variety of agents (Figs. 1 and 2; Tables 1–3). These studies provide further confirmation that the low molecular weight stress protein [later identified as heme oxygenase (7)] that we first observed after treatment of human skin fibroblasts with UVA radiation and the oxidant, H_2O_2 (18), is identical to that observed in both murine and human cells after treatment with the tumor promoter, TPA, heavy metals, and sulfhydryl reagents including sodium arsenite (15–17, 19, 20). Thus it appears that, in addition to the primary role of heme oxygenase in catabolizing the formation of biliverdin from hemoglobin, the enzyme may have an important secondary role in protecting cells against certain types of stress.

A clue to the importance of the transient high level accumulation of heme oxygenase mRNA is to be found in the types of agents which induce the response and which we have broadly classified into two groups (Table 3). The first group (UVA radiation, hydrogen peroxide, ionizing radiation, menadione, and the tumor promoter, TPA) is composed of agents which either are oxidants themselves or are able to generate active intermediates. Since one of the primary effects of ionizing radiation is to generate hydroxyl radical (27) and this intermediate appears to be of central importance in generation of the response by certain agents (28), a larger than 4-fold maximum induction (Table 1) may have been expected. However, the initial induction of mRNA accumulation in the low dose range may be countered by the severe effects of ionizing radiation on macromolecular synthesis that include disruption of RNA transcription. The second category of agents consists of those which are known to interact with or modify cellular glutathione levels (buthionine sulfoximine, sodium arsenite, iodoacetamide, diamide, and cadmium chloride). The effect of modulation of intracellular glutathione levels on the response will be considered in detail elsehwere.[4] However, it is clear that the intracellular redox level strongly influences the magnitude of the inducible response. These observations strongly support the hypothesis that induction of the enzyme is a general response to oxidant stress in mammalian cells and are consistent with the possibility that glutathione plays a key role. The dramatic stimulation of HO mRNA accumulation by UVA radiation (Fig. 2) compared with the low levels of induction after treatment with UVB and UVC radiations at equivalent cytotoxic fluences (Table 1; Fig. 2) is entirely consistent with the concept that radiation at the longer wavelengths constitutes a biologically relevant oxidant stress to cells.

The occurrence of the inducible response in cells cultured from a variety of human tissues that are not involved in hemoglobin breakdown (Fig. 3) further supports the view that the phenomenon represents an important secondary role of heme oxygenase. Since we have observed that lowering cellular glutathione levels substantially enhances the maximum levels of mRNA accumulation observed after a given treatment,[5] the somewhat lower induction in primary epidermal keratinocytes (Fig. 3*A*) may reflect our earlier observations (29) that glutathione levels are 2–3-fold higher in epidermal keratinocytes than in fibroblasts cultured from the same foreskin biopsy. Nevertheless, induction is substantial in all cell types and will lead to a dramatic transient increase in the rate of cellular heme breakdown. Not only free iron but also the iron contained in heme and heme-containing proteins can become available to participate in the Fenton reaction in which active hydroxyl radical is generated from peroxides (30, 31). Furthermore, highly reactive heme-associated ferryl species may be generated by the reaction of certain heme-containing proteins with oxidants (see Ref. 32 for overview). Our current working hypothesis is, therefore, that the strong induction of heme oxygenase by UVA radiation and other types of oxidant stress will play a protective role by rapidly reducing cellular heme pools and thereby suppress the generation of hydroxyl and, possibly, ferryl species under conditions of sustained oxidant stress.

The signal transduction pathways by which oxidant stress and other stimuli lead to increased heme oxygenase gene expression are unknown. Although different pathways are likely to be involved, the increase in mRNA accumulation appears to occur

[4] D. Lautier and R. M. Tyrrell, manuscript in preparation.
[5] D. Lautier and R. M. Tyrrell, unpublished observations.

as a result of changes at the level of RNA transcription both in murine cells after heat shock (33), heme or heavy metal (26) exposure or in human cells after treatment with oxidants or sulfhydryl reagents (8). Genomic clones including upstream flanking sequence for the human gene have been isolated and partially sequenced (Ref. 14)[6] and the search is now under way to determine *cis*-acting genetic elements involved in the inducible responses.

In summary, we have shown that the induction of expression of the heme oxygenase gene is a general response to oxidant stress that occurs, without exception, in cells cultured from a variety of human tissues and mammalian species. Although functional evidence for a protective role is still lacking, the wide distribution of the response and the properties of the enzyme itself encourage the hypothesis that the phenomenon reflects a powerful mechanism by which the prooxidant state of cells can be transiently lowered in order to avoid damage during a sustained oxidative stress.

ACKNOWLEDGMENTS

The authors wish to thank Drs. P. Amstad, J-F. Cajot, H. Tevaearai, and P. Beard for various cell lines.

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[6] Unpublished observations.