Singlet Oxygen: A Primary Effector in the Ultraviolet A/Near-Visible Light Induction of the Human Heme Oxygenase Gene

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ABSTRACT

Both singlet oxygen and the hydroxyl radical are generated in mammalian cells by UVA (320–380 nm) and possibly near-visible (380–420 nm) radiation. We have modulated the cellular levels of these two reactive oxygen species in order to compare their involvement in the induction of the human heme oxygenase (HO) gene by broad spectrum UVA/near-visible light (UVA/NVL). Irradiation in deuterium oxide (in which singlet oxygen has a longer half-life) enhances the broad spectrum UVA/NVL induction of this gene. Sodium azide and l-arginine which are scavengers of both singlet oxygen and the hydroxyl radical reduce the fluorescence-dependent accumulation of HO mRNA, while compounds which are only hydroxyl radical scavengers, namely, mannositol and dimethyl sulfoxide do not. Rose Bengal, a known generator of singlet oxygen, also increases the HO mRNA levels, and this induction is enhanced in deuterium oxide. We conclude that the observed effects of deuterium oxide and singlet oxygen scavengers on HO mRNA levels are not due to a nonspecific effect on transcription but that singlet oxygen is a primary effector in the UVA/NVL induction of the human heme oxygenase gene.

INTRODUCTION

Heme oxygenase (EC 1.14.99.3), the rate-limiting enzyme in the heme degradation pathway, is ubiquitous in higher eukaryotes. Generally, the enzyme is induced in vivo and in vitro by diverse factors such as heme compounds, therapeutic agents, heat shock, hormones, bacterial toxins, sulfhydryl reagents, metal ions, etc. (1). In human skin fibroblasts, HO is induced by UVA (320–380 nm), a component of sunlight clearly established as a carcinogen in animals (2). The human HO gene is also induced by the oxidizing agent hydrogen peroxide (3, 4) but not by heat shock (3, 5, 6). In rat liver, induction of HO enzyme activity by either hemin, cadmium chloride or bromobenzene also caused an increase in the translatable HO mRNA (7). This was the first indication that the induction of HO gene expression was probably by transcriptional activation. Nuclear run-on transcription assays in human skin fibroblasts (8) showed that the gene is induced by a transient increase in the transcription rate of HO mRNA, reaching a maximum within 1 h after treatment and then declining steadily to basal levels by 6 h. The initial increase in the rate of HO mRNA transcription is also correlated with an accumulation of HO mRNA to a maximum between 2 and 4 h, followed by a decrease to basal level by 8 h. Thus, the HO gene induction by UVA involves transcriptional activation, but the signal transduction events are not yet well understood.

In a previous report (9), it was shown that the presence of iron chelators, o-phenanthroline and desferrioxamine, lowered the H2O2 induction of both HO mRNA and protein by 50–80%. A similar effect of these iron chelators was observed on the induction of the HO protein by near-monomochromatic UVA (365 nm) radiation. From this study, it was concluded that the ·OH generated by the iron catalyzed Fenton reaction was involved in the induction of the HO gene. However, iron is also involved in other reactions, such as decomposition of lipid hydroperoxides and autoxidation of a number of biomolecules. Thus, in addition to the ·OH, iron-catalyzed reactions could generate a number of other active oxygen species such as the lipid peroxyl radical and singlet oxygen (10). Furthermore, chemical modulation of UVA inactivation of human fibroblasts (11) indicated that O2 generation plays a major role in the cytotoxicity of UVA. This prompted us to address the question of the involvement of ·OH and O2 in HO gene induction by UVA in more detail.

A common approach to detect O2 involvement in reactions is the use of scavengers. A disadvantage of this approach is that some of the commonly used scavengers of O2 are also efficient scavengers of the ·OH as seen by the in vitro rate constants shown in Table 1 (12, 13). Another approach is to replace water by D2O in which O2 has a longer half-life (14). A combination of the two approaches as used in this study will provide complementary information.

It has been shown that UVA irradiation of macroolecules in vitro causes the generation of H2O2 and superoxide anion (15, 16). Furthermore, it has been proposed that the ·OH could be generated by the iron-catalyzed reduction of H2O2 by superoxide anion (17), and this was demonstrated in vitro (18). Studies of prokaryotic and eukaryotic cells indicate that the ·OH could be generated in vivo by UVA irradiation (15, 16). On the other hand, a number of reactions capable of generating O2 under conditions relevant in vivo have been proposed (19). It has also been suggested that O2 is involved in the UVA-induced lipid peroxidation of liposomal membranes in vitro (20, 21), and lipid peroxidation by UVA has been demonstrated recently in cultured human fibroblasts (22). Since both ·OH and O2 are generated in vivo by UVA irradiation, we have modulated the cellular levels of these active oxygen species by various chemical agents. We find that O2 is a primary effector in the UVA/NVL induction of HO mRNA.

MATERIALS AND METHODS

Cell Culture. The normal human fibroblast cell line FEK4 (23) was cultured at 37°C in Earle’s modified minimal essential medium supplemented with penicillin, streptomycin, glutamine, sodium bicarbonate, and 15% fetal calf serum. Cells were passed at weekly intervals by trypsinization and used for experiments between passages 8 and 17.

Chemical Treatments and UVA Irradiation. All test agents (100 mM sodium azide, 10 mM l-histidine, 0.1 mM mannositol, and 4% DMSO) were dissolved in PBS. D2O buffer solution was prepared by dissolving 1 PBS tablet (Oxoid, Basingstoke, England) in 100 ml of 100% D2O. Cells were seeded (5 × 105/10-cm dish) in 10 ml of medium 3 days prior to the experiment. Prior to treatment with the test agent and UVA irradiation, the culture medium was removed and kept aside. The cell monolayer was rinsed with PBS, 5 ml of the test agent in PBS (supplemented with Ca2+/Mg22+, 0.01% each) was added to each dish and incubated at 37°C for 15 min. Control dishes were incubated for the same time in 5 ml of PBS containing Ca2+/Mg22+. After pretreatment with test agents, cells were irradiated with different fluences (0–1 MJ/m2) of UVA using a broad spectrum (330–450 nm) with maximum energy between 360 and 420 nm) UVASUN 3000 lamp (Mutzhau, Munich, Germany) at a distance which did not cause heating during irradiation. Although this lamp is consid-
ered primarily as a UBS-emitting lamp, we cannot exclude the possibility that the near-visible (380-420 nm) component contributes to the effects observed; hence, the term UVA/NVL is used for the radiation emitted by this lamp. After irradiation, the PBS containing test agent was aspirated, cells were washed with fresh PBS, the original medium was added back, and the cells were incubated for 3 h at 37°C prior to the extraction of total RNA.

**RNA Isolation and Analysis.** Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (24), electrophoresed (15 µg/well) in a 2.2 m formaldehyde-1.3% agarose gel (25) and transferred onto a Gene Screen nylon membrane (NEN Research Products) by capillary blotting (conditions proposed by the manufacturer were used for RNA transfer and hybridization). Baked blots were hybridized to the larger (1 kb) EcoRI fragment of a full-length HO cDNA clone [clone 2/10; (4)] which was 32P labeled by the random-priming DNA-labeling method (26). After autoradiography, the HO probe was stripped off by boiling in 0.1% sodium dodecyl sulfate (twice for 20 min each time), and the blots were reprobed with the 32P-labeled Pfu fragment (1300 base pairs) of rat GAPDH cDNA. The GAPDH-RNA signal was used as an internal control for the loading error between samples since the constitutive levels of this RNA are unaffected by the test agents used.

For autoradiography, films were preflashed, and blots were exposed at -70°C. Radioactive signals were quantified by densitometry on a Elscript 400 (Hirschmann) densitometer. The HO mRNA signals (corrected for the loading error with GAPDH) was expressed as a relative (fold) increase above basal level and plotted as a function of fluence.

The ratio of the corrected HO mRNA area of treated sample and the corresponding control was also calculated for each sample, and mean values of 3–5 experiments for each test agent were plotted as a function of fluence. These plots represent the general pattern of the fluorescence-dependent effect of the test agents.

**RNA Synthesis in Cells.** Cells were cultured in 12-well tissue culture dishes (105 cells/ml well) for 24 h and labeled with [3H]thymidine (10 nCi/well of 57 mCi/mmol specific activity) for another 24 h. After the cells were treated with test agent in PBS (1 ml/well) and UVA/NVL as described above, 1.5 ml of the original culture medium supplemented with 3 µCi of [3H]uridine (specific activity, 46 Ci/mmol) was added back to each well and incubated for 3 h at 37°C. Cells were lysed in 100 µl of lysis buffer [2% sodium dodecyl sulfate-0.1 mg/ml bovine serum albumin-20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3] and spotted onto GF/C discs prewetted with 1 N HCl (27). Discs were washed with 100% ethanol and air dried, and radioactivity was determined in a liquid scintillation counter. The ratio of [3H]Tc cpm was used as a measure of RNA synthesis.

**Modulation of HO mRNA Levels by a Photosensitizer.** A stock solution of Rose Bengal (1 mM), a known generator of •O2, was prepared in DMSO. Cells were seeded in 10-cm dishes and prepared for chemical treatment as described above. For chemical treatment, 5 ml of PBS (supplemented with Ca2+/Mg2+ containing 1 µM RB) was added to each dish, and cells were incubated in the dark for 30 min at 37°C. Control cells were incubated similarly in Ca2+/Mg2+-PBS without the photosensitizer. After the pretreatment, cells were irradiated with a range of fluences (0–1500 J/m2) of broad spectrum (400–700 nm) visible light by placing the culture dishes on a fluorescent light box. After irradiation, the PBS-containing RB was aspirated, the monolayer was washed with fresh PBS, and the original medium was added back to incubate for 3 h at 37°C prior to RNA extraction.

**RESULTS**

**Modulation of UVA/NVL Induction of Heme Oxygenase mRNA by Deuterium Oxide.** UVA/NVL irradiation of human skin fibroblasts induces accumulation of HO mRNA (Fig. 1, A and B). Since both •OH and •O2 are generated in UVA-irradiated cells, we studied the fluence-dependent accumulation of HO mRNA in cells irradiated with UVA/NVL in the presence of D2O, a solvent in which •O2 is known (14) to have a longer half-life. Cells were incubated for 15 min in buffer prepared with 100% D2O prior to irradiation with UVA/NVL fluences ranging from 0–0.5 MJ/m2. We find (Fig. 1, A and B) that in control cells irradiated with UVA/NVL the HO mRNA signal intensity increases steadily to 17-fold above basal level at the maximum fluence used. While treatment with D2O alone does not change the basal level of HO mRNA significantly, the fluence-dependent accumulation of this message is enhanced (Fig. 1, A and B). In D2O-treated cells, the HO mRNA levels first increase to a maximum of 24-fold at 0.2 MJ/m2, and at higher fluences these levels decrease rapidly, reaching basal levels at 0.5 MJ/m2. Ratios of the HO mRNA signal intensities in D2O-treated and control cells (Fig. 1C) show the general pattern of the fluence-dependent D2O effect. We find that the maximum enhancement of the HO mRNA levels is at 0.1 MJ/m2. At higher fluences, the effect declines rapidly, and at 0.5 MJ/m2 the HO mRNA levels in D2O-treated cells are only 10% of the control levels.

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**Table 1 Rate constants for reaction of singlet oxygen and the hydroxyl radical (12, 13)**

<table>
<thead>
<tr>
<th>Test agent</th>
<th>1O2 (M⁻¹s⁻¹)</th>
<th>•OH (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide</td>
<td>2.2 × 10⁶</td>
<td>1.1 × 10⁸</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>3.2 × 10⁷</td>
<td>5.2 × 10⁶</td>
</tr>
<tr>
<td>DMSO</td>
<td>3.4 × 10⁴</td>
<td>7.1 × 10⁴</td>
</tr>
<tr>
<td>Mannitol</td>
<td>&lt;10⁻³</td>
<td>2.7 × 10⁵</td>
</tr>
</tbody>
</table>

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**Fig. 1. Effect of D2O on the HO mRNA accumulation induced by UVA/near-visible light.** Human skin fibroblasts were irradiated with increasing fluences of UVA/NVL in the presence (+) or absence (-) of 100% D2O. A Northern blot of total cellular RNA was first probed for HO mRNA and then for GAPDH mRNA. B, HO and GAPDH mRNA signals were quantified by densitometry. The GAPDH signal was used as an internal loading control. The HO mRNA signal, corrected with GAPDH for difference in loading between samples, was expressed as a relative increase above basal level and plotted as a function of UVA/NVL fluence. C, ratio of the HO mRNA signals (corrected for loading error) in D2O-treated and control cells was calculated for each sample. A mean of 3–5 experiments was plotted as a function of UVA/NVL fluence. Points (bar), mean (±SD).
These results indicate that it is primarily the $^1$O$_2$ generated by UVA/NVL irradiation which is involved in the induction of the HO gene.

**Effect of Sodium Azide and L-Histidine.** To further test the involvement of $^1$O$_2$, we studied the effect of sodium azide (28) and L-histidine (29) which are known to scavenge $^1$O$_2$ almost as effectively as the -OH (Table 1). Monolayer cultures were preincubated with 100 mM sodium azide or 10 mM L-histidine for 15 min and then irradiated with a similar range of UVA/NVL fluences in the presence of the scavenger. The fluence-dependent increase in HO mRNA levels in cells treated with these agents is shown in Fig. 2, A, B, D, and E. These scavengers alone have little effect on the basal level of HO mRNA. In cells treated with sodium azide, the fluence-dependent HO message induction lags initially and reaches corresponding control levels only at 0.4 MJ/m$^2$ (Fig. 2B). L-Histidine also suppresses the fluence-dependent accumulation of HO mRNA (Fig. 2E). The ratios of HO mRNA signal intensity between cells treated with $^1$O$_2$ scavenger and control cells (Fig. 2, C and F) indicate that in the presence of sodium azide the HO mRNA levels are reduced to 40% of the corresponding control at 0.2 MJ/m$^2$ (Fig. 2C). In histidine-treated cells (Fig. 2F), the HO mRNA levels decrease to 50% of the controls at 0.3 MJ/m$^2$. At higher fluences, the HO mRNA levels in cells treated with either scavenger approach that in the corresponding controls.

While these results support those obtained in D$_2$O, the possible involvement of the -OH as a primary effector needed further experimental consideration since the $^1$O$_2$ scavengers mentioned above are also excellent scavengers of the -OH as predicted by their in vitro rate constants (Table 1).

**Hydroxyl Radical Scavengers.** In order to eliminate the possibility that the observed effect of sodium azide or L-histidine on HO induction was really due to scavenging of the -OH radical, we used DMSO (30) and mannitol which scavenge the -OH far more specifically because of their low rate constants for reaction with $^1$O$_2$ (Table 1). We find that these agents themselves have little effect on the basal level of HO mRNA, and the fluence-dependent increase of this message in cells treated with either 4% DMSO or 0.1 M mannitol is similar to that observed in the corresponding control cells (Fig. 3, A, B, D, and E). Plots of fluence-dependent change in ratios (Fig. 3, C and F) indicate that the HO mRNA levels in the -OH scavenger-treated cells are either almost equal to the corresponding controls (in mannitol) or slightly higher (in DMSO) than the levels in the corresponding untreated cells.

Thus, scavengers of the -OH do not decrease the fluence-dependent accumulation of HO mRNA, and at least in cells treated with DMSO cause a slight enhancement of the HO mRNA levels.

**Effect of Test Agents on Total RNA Synthesis.** In order to determine whether the modulation of HO mRNA levels by the various test agents is due to a general effect on transcription, we measured the total $[^3]$H]uridine incorporation during 3 h in cells which had been pre-treated with D$_2$O, sodium azide, or DMSO and then irradiated with increasing fluences of UVA/NVL in the presence of these agents. In control cells, the RNA synthesis is inhibited with increasing fluences and reaches almost zero at 1 MJ/m$^2$ (Fig. 4A). In cells treated with D$_2$O, there is further suppression of transcriptional activity at fluences above 0.25 MJ/m$^2$. In contrast, sodium azide seems to protect against the toxic effects of UVA on transcription, as seen by the higher incorporation of $[^3]$H]uridine in cells irradiated in the presence of azide (Fig. 4B). Finally, the -OH scavenger DMSO does not have a significant effect on the fluence-dependent decrease in transcription (Fig. 4C).

![Figure 2](https://example.com/figure2.png)  
**Fig. 2.** Effect of sodium azide (NaN$_3$) and L-histidine on UVA/NVL-induced HO mRNA accumulation. Northern blot analysis of HO and GAPDH mRNA in the presence (+) or absence (−) of either 100 mM sodium azide (A) or 10 mM L-histidine (D). B and E, HO mRNA signals quantified and corrected for loading error and expressed as a relative increase over basal levels. C and F, ratios of the HO signal intensity in scavenger-treated and control cells plotted as a function of UVA/NVL fluence. Point (bar), mean (±SD).
SINGLET OXYGEN INDUCTION OF THE HUMAN HO 1 GENE

These results indicate that the enhancement of HO mRNA accumulation in D2O and suppression of this accumulation in sodium azide are not due to a general effect of these agents on transcription.

Modulation of HO mRNA Levels by Rose Bengal. It is known (31) that the photosensitizer RB generates ¹⁰₂ when illuminated with visible light. Therefore, we studied the effect of this photosensitizer on HO mRNA accumulation. We find that irradiation with a range of fluences of visible light alone does not induce HO (Fig. 5, A and C). In cells treated with 1 μM RB, the HO mRNA levels increase to 29-fold above basal levels at the maximum fluence of visible light used. In the presence of D2O, the RB/visible light-induced increase in HO mRNA levels is enhanced; the HO message levels increase to 82-fold above basal level at 1 kJ/m² (Fig. 5, B and C). At higher fluences, the HO mRNA levels decline rapidly to basal levels. Thus, the enhancement of HO mRNA accumulation by RB/visible light in the presence of D2O is similar to that observed in the UVA/NVL-induced accumulation of this message.

These results are entirely consistent with the conclusion that it is the ¹⁰₂ which is a primary effector in the UVA/NVL induction of the human heme oxygenase gene.

DISCUSSION

In the present study, we have compared the relative involvement of ¹⁰₂ and .OH in the UVA/NVL induction of the HO gene by chemically modulating the cellular concentration of these active oxygen species, and we find that ¹⁰₂ is a primary effector in this induction. An accompanying observation is that ¹⁰₂ also appears to be the active intermediate involved in the UVA/NVL fluence-dependent inhibition of overall transcription. Since it was not possible to measure effective intracellular concentrations of the test agents used, we used the optimum concentrations as determined in a previous study (11) on radical involvement in UVA inactivation of human fibroblasts.

The UVA/NVL fluence-dependent increase in HO mRNA levels is initially enhanced in D2O (Fig. 1). Although the enhancement of lifetime of ¹⁰₂ in vitro by D2O is by a factor of 10–15, this is not reflected in the enhancement of HO mRNA levels in vivo presumably due to the interaction of this active oxygen intermediate with biomolecules. Furthermore, deuteration of biological antioxidants could contribute to lowering the threshold of UVA/NVL fluence required to induce HO gene in the presence of D2O. At fluences higher than 0.2 MJ/m², the HO mRNA levels decrease rapidly (Fig. 1, A and B) probably due to a further suppression of the UVA/NVL fluence-dependent decrease in total RNA synthesis in the presence of D2O (Fig. 4A). We have observed in untreated cells (data not shown) that at fluences above 0.5 MJ/m², the HO mRNA levels also decline, indicating that severe oxidant stress shuts off transcription of this gene, probably because of a general inhibitory effect on total RNA synthesis in cells. In the presence of sodium azide and L-histidine, the fluence-dependent induction of HO mRNA is significantly (50–60%) lower than the controls, and this is not due to an inhibitory effect on transcription (Figs. 2 and 4B). Thus, in the fluence range in which D2O has no effect on total RNA synthesis, it enhances the UVA/NVL induction of HO mRNA. On the other hand, sodium azide protects to some extent against the toxic effects of UVA/NVL on total RNA synthesis in cells but decreases the UVA/NVL fluence-dependent HO mRNA accumulation up to fluences of 0.3 MJ/m². At higher fluences, these agents are no longer effective, probably because of the generation of radicals by the scavengers themselves. Since sodium azide and L-histidine scavenge both the .OH and ¹⁰₂ with equal efficiency as predicted by their in vitro rate constants (Table 1), our results could be
to scavenging of either one or both of these species. However, DMSO and mannitol which preferentially scavenge the -OH (Table 1) have little or no effect on either the fluence-dependent increase in HO mRNA levels or total RNA synthesis in cells (Figs. 3 and 4C). The possibility exists that the -OH scavengers do not reach the target molecules within the cell. However, for UVA/NVL induction of the HO gene, the cell membrane is a more likely site for triggering a cascade of signal transduction events since UVB and UVC radiations (3, 32), which cause more DNA damage than UVA radiation, do not induce this gene. Any -OH generated by UVA/NVL at or close to cell membranes would be expected to be easily accessible to scavengers. Use of RB (which is known to generate $^{1}O_{2}$) to modulate HO mRNA levels supports the idea of $^{1}O_{2}$ involvement. Interpretation of results obtained using RB is complicated by the fact that it can generate radicals (type I reactions) in addition to $^{1}O_{2}$ (type II reactions). To avoid this problem, we have also induced HO with RB/visible light in the presence of $^{18}O_{2}$ in which type I reactions are presumably unaffected (33). The $^{18}O_{2}$ enhancement of the increase in HO mRNA levels by RB/visible light confirms the effectiveness of $^{1}O_{2}$ in the induction of the HO gene. Furthermore, in the presence of L-histidine, which has little reactivity toward the superoxide anion (28), there is a decrease in the HO mRNA levels induced by RB/visible light (data not shown). Taken together, these results are consistent with the conclusion that it is the $^{1}O_{2}$ which is the primary effector in the UVA/NVL induction of this gene in human skin fibroblasts.

An indication that a crucial early step in HO gene induction could be a membrane event is that porphyrins, which constitute an important class of endogenous photodynamic sensitizers, cause oxidative stress by generating $^{1}O_{2}$ (34) and are present in cell membranes. Moreover, it has been demonstrated in Propionibacterium acnes (35) that the maximum sensitivity to blue light (380–440 nm) was in the region of 415 nm, which corresponded to the absorption maxima of porphyrins in cells. A porphyrin involvement has also been suggested (36) in near UV (300–400 nm) inactivation events in Escherichia coli. It has been shown in bacteria that RB accumulates in the cell membrane (37) and that the photodynamic inactivation of bacteria by this dye is similar to that caused by use of chemically pure $^{1}O_{2}$ on bacterial cells (38). Thus, the RB-mediated photodynamic inactivation seems to be a membrane event in bacteria, probably because of the generation of $^{1}O_{2}$. If we assume that RB also accumulates in the cell membrane of mammalian cells, the HO induction in the presence of this dye and visible light could be interpreted to be a membrane event. There is ample evidence that UVA can cause lipid peroxidation of cell membranes in human skin fibroblasts (22) and, based on experiments with a wide variety of scavengers, this process is believed to be mediated by $^{1}O_{2}$ (20, 21).

Thus, we propose that $^{1}O_{2}$ may be involved as a crucial intermediate at an early step in the signal transduction events that lead to the UVA/NVL induction of the human heme oxygenase gene.

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