Carcinogenesis vol.13 no.2 pp.227–232, 1992

Endogenous glutathione levels modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene

Dominique Lautier, Patrick Luscher and Rex M.Tyrrell[1]

Swiss Institute for Experimental Cancer Research, Ch. des Boveresses 155, CH-1066 Epalinges/Lausanne, Switzerland

[1]To whom correspondence should be addressed

Induction of the expression of the mammalian heme oxygenase gene appears to be a general response to oxidant stress. In view of the role of glutathione in protecting cells against solar UVA radiation and other forms of oxidant stress, we have investigated the relationship between intracellular glutathione levels and the inducibility of the human heme oxygenase gene after treatment of populations of cultured skin fibroblasts with either UVA radiation or hydrogen peroxide. We observe a clear relationship between cellular glutathione status and both the constitutive and oxidant-inducible accumulation of heme oxygenase mRNA. Glutathione depletion may lead to enhanced gene expression either as a result of the potentiated accumulation of active oxygen intermediates or as a result of the direct influence of glutathione on a critical target involved in signal transduction.

Introduction

Solar UVA (320–380 nm) radiation appears to constitute an important oxidative stress to cells (for reviews, see refs 1 and 2) and has the potential to generate a range of active oxygen intermediates intracellularly. This is reflected in observations that most of the biological effects of UVA radiation, ranging from inactivation of colony-forming ability of cultured prokaryotic and eukaryotic cells (3,4) to the skin erythema response in humans (5) are enhanced in the presence of oxygen. Thus it follows that cellular protection against the damaging effects of UVA radiation will involve antioxidant defence pathways.

Intracellular glutathione, which is present in most cell types in millimolar concentrations, is believed to be an important cellular antioxidant compound (6). This hypothesis has been strengthened by observations that depletion of intracellular glutathione sensitizes cell populations to aerobic ionizing radiation (for review, see ref. 7), cytotoxic drugs (8,9) and hydrogen peroxide (H_2O_2) (unpublished observations, this laboratory). Furthermore, populations of cultured human skin fibroblasts or epidermal keratinocytes that have been depleted of glutathione by overnight treatment with low concentrations of D,L-buthionine-*S*,*R*-sulfoximine (BSO*) are strongly sensitized to the lethal action of both UVA and UVB (290–320 nm) radiations (10,11). These results have led us to propose that endogenous glutathione provides a powerful constitutive mechanism of defence against solar UVA radiation.

In more recent studies, we have identified a 32 kDa protein induced by UVA radiation and hydrogen peroxide (12) as the heme catabolic enzyme, heme oxygenase (HO) (13) and shown that control of gene expression is almost entirely at the level of transcription (14). Reports from various laboratories are consistent with the conclusion that HO is the low mol. wt stress protein induced in a range of mammalian cell types by a variety of chemical treatments including heavy metal salts, the sulfhydryl reagent, sodium arsenite and the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (15–18). Although the functional significance of this inducible response is not yet proven, our current hypothesis is that the phenomenon underlies a general inducible antioxidant defense pathway. The transient synthesis of a high level of HO will lead to a rapid reduction in cellular levels of heme and heme-containing compounds, and we propose that the consequent lowering of the prooxidant state of cells will alleviate the deleterious consequences of a sustained oxidant stress. Additional support for the proposal that oxidant stress is an important factor in the stimulation of HO gene expression is the observation that the hydroxyl radical is a key intermediate in the induction observed after UVA or peroxide treatment (19).

Although previous reports have indicated that the glutathione status of the cell does not influence accumulation of the low mol. wt stress protein (12,20,21), GSH depeletion clearly did lower the threshold dose for induction by UVA or peroxide treatment (12). In view of the central importance of glutathione as a cellular antioxidant, we have further investigated the influence of cellular glutathione status on HO gene expression using the greater sensitivity allowed by the analysis of a specific mRNA species. We show that not only is the level of inducible HO mRNA accumulation strongly influenced by the cellular levels of glutathione but also that the level of gene expression correlates with cellular glutathione levels in the absence of inducing treatment.

Materials and methods

All chemicals and biochemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). Adenosine-5′-([α-^{32}P]triphosphate) was obtained from Amersham (UK).

Cell culture and treatments

The human skin fibroblast cell line FEK4 was derived from a foreskin explant and cultured routinely as described previously (22). A total of 1.5×10^6 cells were seeded per 200 mm diameter plastic dish 3 days prior to chemical treatment or UVA irradiation. For both UVA and H_2O_2 treatments, medium was removed and reserved and cells were rinsed with isotonic PBS. H_2O_2 was added in phosphate buffer for 30 min at 37°C. For UVA irradiation, the monolayer was covered with a solution of PBS containing Ca^{2+} and Mg^{2+} and was irradiated at 365 nm using a broad-spectrum (340–450 nm) Uvasun 3000 lamp (MUTZHAS, FRG). The irradiance was monitored with an IL1700 radiometer. Maximum irradiation times were <45 min. Control dishes were sham-irradiated. After treatment, PBS was removed and the conditioned medium was added back to the cells for various times prior to isolation of total RNA.

In certain experiments, BSO, an inhibitor of γ-glutamylcysteine synthetase, was added to the culture medium 18 h prior to treatment in order to deplete glutathione. Under the conditions employed, this drug showed no toxicity at least at concentrations as high as 500 μM.

Isolation of total RNA

Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform extraction method (23). For each sample, 2 μg of total RNA were loaded onto a Tris–borate–EDTA (TBE) agarose gel (1%) to control quality and quantity.

*Abbreviations: BSO, D,L-buthionine-*S*,*R*-sulfoximine; HO, heme oxygenase; GSH and GSSG, reduced glutathione and its oxidized form (glutathione will be written in full where the two forms are not distinguished analytically); NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DEM, diethylmaleate.

Fifteen micrograms of total RNA were then loaded onto a MOPS/formaldehdye agarose gel (1.3%), electrophoresed, transferred to a sheet of Genescreen (NEN Research Products) and hybridized to a ^{32}P-labeled DNA probe prepared by random primed synthesis of a full-length cDNA (1.4 kb) of the human HO gene (clone 2/10, ref 13). Levels of mRNA were quantitated by densitomery, using an Elscript 400 (Hirschmann) densitometer. Data were expressed as the ratio of the level of the appropriate mRNA in treated cells to that in the untreated control. The results are expressed as the means ± SD of at least three determinations. For untreated control cells and cells treated only with BSO, HO mRNA levels were determined in 20 independent experiments.

Glutathione measurements

After treatment of cell monolayers, extracellular medium or PBS was removed and stored at −20°C for subsequent determination of extracellular glutathione concentrations. Cell monolayers were then rinsed with PBS, trypsinized, resuspended in cold PBS, counted electronically and pelleted by centrifugation at 4°C. The cell pellets were extracted with a freshly prepared mixture of 5% TCA and 2 mM EDTA, in order to give 1 ml of extract per 2 × 10^6 cells. After centrifugation at 4°C, each supernatant was divided into two aliquots in order to measure both total intracellular glutathione, and the oxidized form, glutathione disulfide (GSSG), using the spectrophotometric method adapted from Tietze (24). *n*-Ethylmaleimide (NEM) (50 mM final) was added in the samples for GSSG determination, and incubation was for 1 h at 25°C. NEM was completely removed using 10 extractions with ether, prior to the GSSG reductase recycling assay using 5,5'-dithio-bis(-2-nitrobenzoic acid). The data are expressed as the percentage of total intracellular GSH equivalents and are means ± SD of three or four independent experiments. The extracellular glutathione measured in these assays is a mixture of GSH, GSSG and possibly mixed disulfides that are reduced via sulfhydryl−disulfide exchange reactions.

Results

Glutathione depletion with BSO

Figure 1 shows the correlation between the levels of intracellular and extracellular glutathione—reduced (GSH) and oxidized (GSSG) forms—and increasing concentrations of BSO. The results are expressed as a percentage of the total GSH + GSSG concentration in control cells, which is 5.15 ± 1.1 nmol GSH equivalents per 10^6 cells, while extracellular glutathione and intracellular GSSG are 65 and 4−6% of the total intracellular pool respectively. The values represent the concentration of glutathione measured 18 h after a complete medium change. Incubation of cell populations with various concentrations of BSO for 18 h leads to a concentration-dependent depletion of intracellular glutathione levels. The reduced levels of total intracellular glutathione are accompanied by a concomitant decrease in the intracellular GSSG and exported glutathione levels.

For BSO concentrations of 20 μM or higher, the total intracellular GSH + GSSG level falls below 5% of the level in untreated cells. However, since the concentration of intracellular GSSG falls in parallel with that of total GSH + GSSG as the BSO concentration is increased, the assay for intracellular GSSG becomes increasingly less reliable. The decrease in extracellular glutathione is in agreement with the observation of a decrease in glutathione export after BSO-induced glutathione depletion (25). No noticeable change in the intracellular GSSG/GSH ratio has been observed as long as intracellular GSSG is detectable.

Based on preliminary experiments, BSO (18 h incubation) at a concentration of 50 μM has been used to deplete glutathione in the experiments that follow except where otherwise stated.

The influence of incubation time on induction of HO mRNA accumulation in normal and glutathione-depleted cell populations

In order to characterize the time-course of HO induction after UVA or H_2O_2 treatment, Northern analyses have been carried out at different times after treatment. It has been shown previously that maximal induction of HO mRNA accumulation by UVA or H_2O_2 treatment is observed 3−4 h after treatment (13) and this is now confirmed by the data shown in Figure 2(A) and (B). After 8 h, the level of mRNA accumulation approaches control values. The large variation in the fold increase in HO mRNA levels seen in these and subsequent experiments appears to be due, in large part, to the considerable variation in constitutive messenger levels from experiment to experiment.

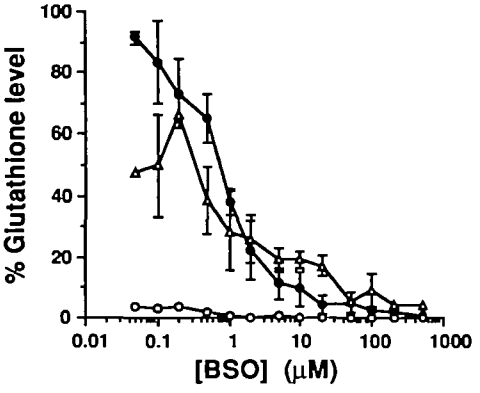

Fig. 1. Modulation of total intracellular GSH + GSSG, intracellular GSSG and extracellular glutathione by BSO treatment. Cell populations have been treated with various concentrations of BSO for 18 h prior to glutathione measurements. The results are expressed as percentage of total intracellular glutathione levels measured in untreated cells. The control values are 5.15 ± 1.1 nmol GSH equivalent/10^6 cells for total intracellular glutathione (closed circles) and 4−6% and 65% of this value for intracellular GSSG (open circles) and total extracellular glutathione (open triangles) respectively.

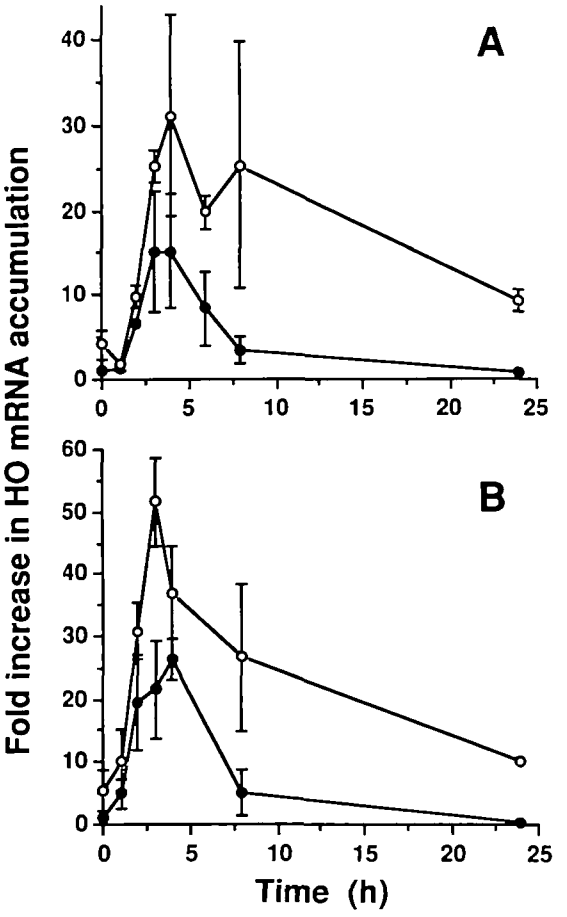

Fig. 2. Kinetics of induction of HO mRNA accumulation after (**A**) UVA irradiation at a fluence of 2.5 × 10^5 J/m^2 and (**B**) incubation with 50 μM H_2O_2 for 30 min either in normal cells (closed circles) or in cells depleted of glutathione by overnight (18 h) pretreatment with 50 μM BSO (open circles).

Preincubation of cells wtih BSO leads to a significant enhancement in the level of HO mRNA accumulation and also lengthens the period over which mRNA accumulation remains high. Twenty-four hours after either UVA or H_2O_2 treatment, HO mRNA levels in BSO-treated cells are 10-fold higher than in cells not treated with BSO.

The influence of radiation fluence and hydrogen peroxide concentration on accumulation of HO mRNA in normal and glutathione-depleted cell populations

Figure 3(A) and (B) show the levels of HO mRNA accumulation over a range of fluences of UVA radiation or increasing H_2O_2 concentrations. Based on the data shown in Figure 2, total RNA was extracted after 3 h post-treatment incubation. Both UVA irradiation and incubation with H_2O_2 induce a dose-dependent increase in HO mRNA accumulation. Under these treatment conditions, up to 20-fold increases in HO mRNA accumulation were observed for both agents. However, in glutathione-depleted cells, the pattern of HO mRNA accumulation differs between the UVA radiation and H_2O_2 treatment. While BSO treatment strongly enhances H_2O_2-induced mRNA levels throughout the concentration range tested (~ 100-fold over the control value for the highest H_2O_2 concentrations tested), the enhanced mRNA accumulation in UVA-irradiated cells only occurs at fluences $< 5 \times 10^5$ J/m^2. This would appear to be related to the toxic effect of UVA radiation that occurs as a result of extreme glutathione depletion (10,11).

Another interesting result to emerge from these experiments is evident from the control points (fluence = 0) on Figure 3(A) from which it is clear that BSO treatment alone can lead to an increase in HO mRNA accumulation. Indeed in 20 independent experiments (results not shown) there is an average of 5.4 ± 2.3-fold increase in HO mRNA accumulation resulting from overnight incubation with BSO. This result was unexpected in view of previous studies with glutathione-depleted cells in which no detectable increase in levels of the 32 kDa protein were observed (12,20,21).

The influence of glutathione level on the induction of HO mRNA accumulation after UVA or H_2O_2 treatment

In order to determine the relationship between cellular glutathione status and levels of induced mRNA accumulation, cells have been treated with various concentrations of BSO (0.05 − 500 μM) for 18 h, prior to either UVA irradiation at a fixed fluence of 2.5×10^5 J/m^2 (Figure 4A), or 30 min incubation with 50 μM H_2O_2 (Figure 4B). Such conditions will lead to 4-fold and 8-fold increases (relative to control) in mRNA accumulation after UVA irradiation and H_2O_2 treatment respectively. In the absence of any inducing treatment, a BSO concentration as low as 1 μM is able to induce a 2-fold increase in HO mRNA accumulation. As shown in Figure 1, this drug concentration decreases the total intracellular glutathione pool to 40% of the value in untreated cells. At this concentration of BSO, the effects of glutathione depletion and UVA radiation/H_2O_2 treatment appear to be cumulative. For higher concentrations (up to 50 μM BSO), induction of mRNA is strongly increased in UVA- or H_2O_2-treated cells. However, pretreatment with BSO at a still higher concentration leads to a drop in UVA-induced HO mRNA levels. This is presumably again due to a cumulative toxic effect

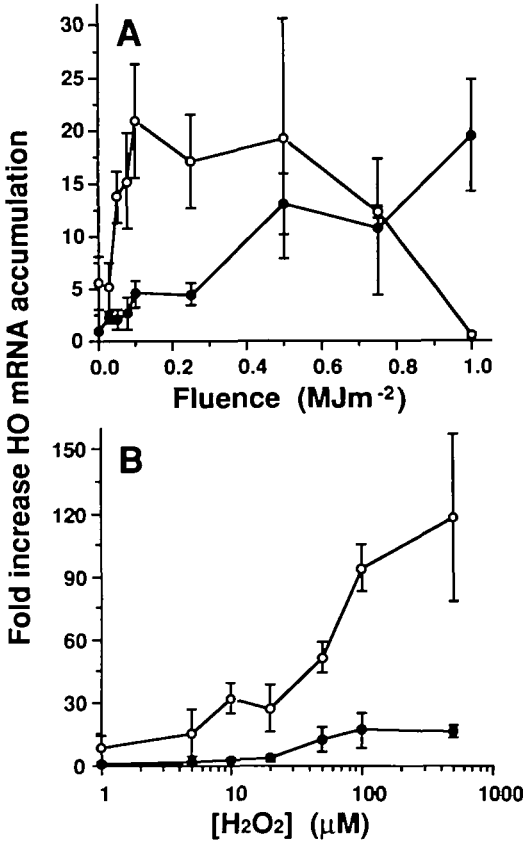

Fig. 3. Induction of HO mRNA accumulation as a function of (A) increasing fluences of UVA radiation and (B) increasing H_2O_2 concentrations (30 min exposure). RNA extractions have been carried out 3 h after UVA or H_2O_2 treatment of normal cells (closed circles) or cells depleted of glutathione by overnight incubation with 50 μM BSO (open circles).

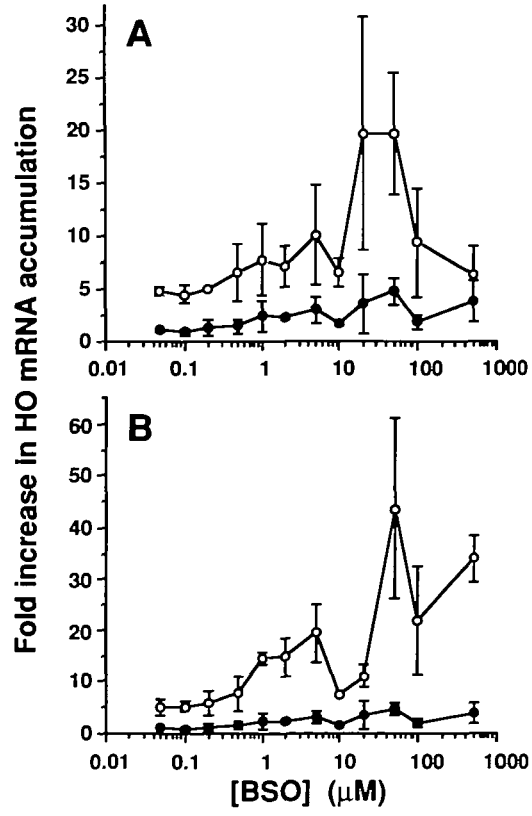

Fig. 4. The influence of an 18 h exposure to a range of concentrations of BSO on HO mRNA accumulation in the presence (open circles) or absence (closed circles) of an inducing treatment with (A) UVA at a fluence of 2.5×10^5 J/m^2 or (B) 50 μM H_2O_2 for 30 min.

of the radiation and BSO, since gutathione levels are maximally depleted at 20 μM (Figure 1). Pretreatment of cell populations with BSO at these higher concentrations does not lead to a decline in H_2O_2-induced HO mRNA levels (Figure 4B).

Glutathione measurements in UVA-irradiated or H_2O_2-treated cells

Since depletion in glutathione levels by the oxidants themselves may also influence levels of mRNA accumulation, we have measured changes in intracellular and extracellular glutathione levels 30 min after treatment of cell populations with either UVA radiation (Figure 5A) or H_2O_2 (Figure 5B). Figure 5(A) shows that glutathione is increasingly depleted as a function of UVA fluence after an initial plateau at lower fluences. At a fluence of 5×10^5 J/m^2, <40% of total intracellular glutathione remains in the irradiated cells. At the same time, the proportion of glutathione exported is increased over that in untreated cells (7.3 ± 3.2% of total intracellular glutathione) and can account for a significant fraction of the loss in intracellular glutathione. Intracellular GSSG is undetectable at high fluences.

As shown in Figure 5(B), H_2O_2 concentrations in the range of 0.1−20 μM (30 min treatment) are not sufficient to lower the glutathione pool. Above 20 μM, there is a decrease of total intracellular glutathione and an increase in extracellular glutathione. Within the range of H_2O_2 concentrations used, the fall in total intracellular glutathione is lower than that observed after UVA radiation and represents 50% of the control value for the highest H_2O_2 concentration used (1 mM), while the extracellular glutathione level reaches 45% of the total intracellular glutathione. Thus, the export of glutathione appears to account for most of the reduction in intracellular glutathione that results from H_2O_2 treatment.

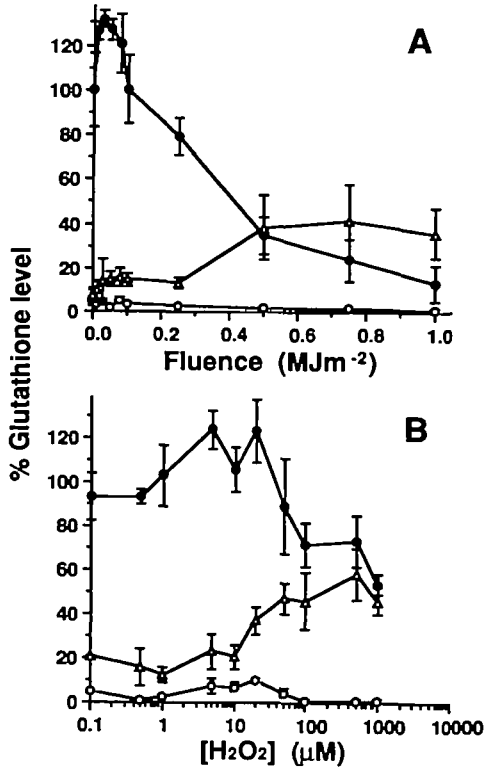

Fig. 5. Modulation of glutathione levels by (A) UVA treatment or (B) H_2O_2 treatment. Results are expressed as a percentage of the total intracellular GSH + GSSG level, as measured after incubation of untreated cells for 30 min in PBS. Total intracellular GSH + GSSG (closed circles); intracellular GSSG (open circles); extracellular glutathione (open triangles). Control values are 5.5 ± 0.9 nmol GSH equivalent/10^6 cells for total intracellular glutathione, and 4.6 and 7.3% of this value for intracellular GSSG and extracellular glutathione respectively.

Discussion

The central observaton emerging from the current study is that depletion of the level of intracellular glutathione by treatment of cells with BSO (Figure 1) is correlated with an enhanced accumulation of HO mRNA both in cells that have been subsequently treated with inducing agents (UVA and H_2O_2) and in cells that have not received the additional inducing treatment (Figures 2−4). The latter result was somewhat unexpected in view of previous data from laboratories, including our own, which failed to show an increase in levels of a 32 kDa protein—later shown to be HO (13)—following depletion of cellular glutathione with BSO (12,20,21). On the other hand, Shelton *et al.* (20), Freeman and Meredith (21) and more recently Taketani (26) have observed that diethylmaleate (DEM), which also depleted glutathione, is an effective inducer of the protein. While BSO reduces GSH levels by inhibition of *de novo* synthesis, the reduction by DEM occurs as a result of both GSH conjugation and mixed-disulfide efflux. These observations led to the conclusion that both GSH depletion and GSH conjugation are required for accumulation of the protein. However, the findings reported in the present study, which measure HO gene expression by mRNA accumulation rather than the appearance of the protein, are clear evidence that glutathione depletion alone is sufficient to modulate gene expression. Even a partial glutathione depletion (60%), observed after treatment of cells with BSO at a concentration as low as 1 μM, induces a 2-fold increase in HO mRNA accumulation (Figure 4).

Since it appears likely that the mRNA accumulated after glutathione depletion by BSO is translated, the failure to observe enhancement of the 32 kDa protein in previous studies is almost certainly a question of sensitivity. Many proteins of ~32 kDa are constitutively expressed in human cells. A 4- to 5-fold enhancement in one of the proteins may not be detectable by one-dimensional SDS−PAGE, the technique normally employed to measure increased levels of the 32 kDa protein.

Several different experiments (Figures 2−4) show that the induction of HO mRNA accumulation by UVA radiation or H_2O_2 is significantly potentiated by depletion of cellular glutathione. The maximal levels of mRNA accumulation occur 2−3 h after treatment and at that time the fold enhancement is apparently 2 at the UVA radiation fluence (2.5×10^5 J/m^2) and H_2O_2 concentration (50 μM for 30 min) selected (Figure 2A and B). However, considering the overall fluence/dose−response curves for UVA- and H_2O_2-induced mRNA accumulation in non-depleted or glutathione-depleted cells (Figure 3A and B), the fold enhancement resulting from glutathione depletion appears to be closer to a factor of 4. The picture for the UVA radiation-induction of HO mRNA accumulation is complicated by a competing reaction that leads to a decline in accumulation in cells that have been depleted of glutathione and irradiated with UVA fluences $>5\times10^5$ J/m^2 (Figure 3A). This is almost certainly related to our previous observations that under conditions of glutathione depletion, human fibroblast populations become extremely sensitive to UVA radiation even at fluences as low as 10^5 J/m^2 (10). Toxicity at high BSO concentrations could also explain why maximal potentiation of UVA-induced HO mRNA accumulation is observed in the range of BSO concentrations from 10 to 50 μM (Figure 4A). The potentiation of H_2O_2-induced mRNA accumulation by glutathione depletion continues to rise

over the range of H_2O_2 (Figure 3B) and BSO (Figure 4B) concentrations employed.

Both UVA (for review, see ref. 2) and H_2O_2 have the potential to generate active oxygen intermediates in cells. Glutathione constitutively protects cells against oxidative damage so that lowered cellular glutathione levels would be expected to lead to a diminished potential to reduce/detoxify active intermediates (27), which in turn could lead to the potentiation of the mRNA accumulation resulting from UVA/H_2O_2 treatment. Furthermore, if the primary role of glutathione in enhancing HO gene expression involves altered levels of active intermediates, then the differences in mRNA accumulation observed between normal and glutathione-depleted populations that have not been treated with oxidants are indicative that cellular metabolic activity alone generates sufficient active intermediates to modulate gene expression. Although this is a viable possibility, an alternative possibility, that glutathione levels may be more directly involved in regulating gene expression, should also be considered. For example, lowering cellular reducing potential could lead to the conformational alteration of a protein factor involved in transcriptional regulation of the HO gene. There is evidence *in vitro* that transcriptional activity mediated by protein factors binding to DNA may be regulated by a redox mechanism (see ref. 28 for example and references therein). Another possibility is that membrane alterations play a key role in the signal transduction pathway. Low glutathione levels may compromise the activity of GSH peroxidase and other GSH-dependent enzymes and lead to enhanced peroxidation of membrane lipids (29,30). The damage will be further exacerbated when additional stress is provided by oxidants such as UVA radiation (11) or H_2O_2. Furthermore, glutathione is known to be involved in regulation of membrane permeability, and a high GSH/GSSG ratio is essential for maintenance of sulfhydryl proteins in the reduced state in order to control Ca^{2+} release (31). The rapid membrane changes that result from altered cellular glutathione status should certainly be considered as potential signaling events in the stress response pathway.

A further indication that intracellular glutathione plays a crucial role in the stress response is that both UVA radiation and H_2O_2 lead to reductions in intracellular levels of glutathione (Figure 5). In the case of H_2O_2, most of the disappearance of glutathione can be accounted for by cellular efflux. However, this is not so for high fluences of UVA radiation, which may involve conjugation of activated molecules to GSH. These observations are again consistent with the possibility that it is the change in cellular GSH status rather than the enhanced generation of active oxygen intermediates that is primarily responsible for the induction of the stress response. Certainly, the induction of HO mRNA accumulation by UVA radiation and H_2O_2 (Figure 3A and B) occurs in the same fluence and concentration range over which cellular glutathione levels are being lowered. However, the current experiments do not provide a sufficient basis for distinguishing the primary pathways that lead to induction of expression of the HO gene by oxidants.

In summary, we have shown that alteration of cellular glutathione status by a highly specific inhibitor of GSH synthesis is sufficient to strongly modulate levels of HO mRNA expression. Lowered glutathione levels not only enhance the induction of gene expression under conditions of oxidative stress (UVA radiation or H_2O_2) but also alter basal levels of expression. These findings further support our hypothesis that induction of this gene is an important response to oxidant stress. However, additional studies are required to determine whether lowered glutathione levels enhance the inducible response simply by allowing the accumulation of increased levels of active oxygen intermediates or whether glutathione exerts its influence by modulation of a signaling mechanism such as an alteration in the reduction state of a critical protein or alteration of membrane integrity.

Acknowledgements

The work reported in this paper has been supported by grants from the Swiss National Science Foundation (3.186.088) and the Swiss League Against Cancer.

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Received on July 22, 1991; revised on October 21, 1991; accepted on November 11, 1991