Temperature-dependent Influence of Thiols upon Glutathione Levels in Chinese Hamster Ovary Cells at Cytotoxic Concentrations

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ABSTRACT

Chinese hamster ovary cells were exposed to the sulfhydryl compound cysteamine at different temperatures (5°C, 37°C, 44°C) at concentrations known to generate activated oxygen species. At 37°C, the cellular glutathione (GSH) content increased linearly over the time of drug exposure (2 h) as compared to untreated cells or to cells kept at 5°C during drug treatment. The 2- to 4-fold increase in GSH induced by cysteamine was more rapid at 44°C than at 37°C and showed a saturation effect at the higher temperature. The elevation of GSH could be completely blocked by dl-buthionine-S,R-sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, or by incubation in a cystine-free medium during the period of drug treatment. The increased cellular GSH content induced by cysteamine alone at 37°C or combined with heat at 44°C decreased to the range of control values within 22 h after either treatment. Other thiols like cysteamine, namely cysteine, L/-acetylcysteine, and dithiothreitol, were found to be similar in their potential to induce GSH elevation in Chinese hamster ovary cells. Cytotoxic effects of these sulfhydryl compounds were observed in the same concentration range as that for cysteamine (0–2 mm), but only if cells were plated at low densities (10^5–10^6 cells/flask), and were completely blocked by the addition of catalase (50 μg/ml). In contrast, the elevation of GSH after thiol treatment (0.8 mm) was not modified by catalase. The data suggest that thiol treatment combined with hyperthermia leads to a rapid increase of GSH biosynthesis in Chinese hamster ovary cells which seems to be independent of the simultaneous generation of activated oxygen species by thiol autoxidation.

INTRODUCTION

Rapid elevation of GSH, a tripeptide involved in maintenance of the cellular oxidation-reduction potential (1), has been reported as cellular response to thermal stress (2). GSH has many roles in cells (3), including regulation of thiol oxidation-reduction status and protection against cellular damage by electrophilic or free radical agents. Conjugation of GSH with chemically reactive intermediates (4) and glutathione disulfide formation during oxidative challenge can cause GSH depletion (5). The rate limiting step in GSH biosynthesis may be the availability of cysteine (6). It has been shown previously that the cytotoxicity of cysteamine is enhanced by heat (7, 8) and that activated oxygen species play an important role in the thermosensitization of this drug (8, 9). We also have found that thiols like cysteamine consume oxygen and produce hydrogen peroxide under hyperthermic conditions and that the reaction rate is dependent upon environmental factors (pH, metal ions) (10).

The purpose of this study was to measure the cellular GSH content after oxidative stress by exposure of Chinese hamster ovary cells to thiols like cysteamine at different temperatures.

MATERIALS AND METHODS

Cell Culture. CHO cells were routinely cultured in McCoy’s Medium 5A supplemented with 10% (v/v) newborn calf serum and 5% (v/v) fetal calf serum, penicillin (0.05 g/liter), streptomycin (0.05 g/liter), and neomycin sulfate (0.1 g/liter). Cells were maintained in exponential growth at 37°C in a 5% CO2 atmosphere. Under these conditions, the population doubling time of exponential phase cells was approximately 14 h, and colony forming efficiency was 80 to 90% (11). The cells were routinely subcultured every 2 or 3 days.

In Vitro Drug Exposure. It should be emphasized that cell density during drug exposure in experiments designed to measure changes in GSH of CHO cells (x10^6 cells/flask) differed from experiments to determine the influence of these compounds upon cell survival (10^5–10^6 cells/flask). However, the experimental protocol described here for exposure of cells to cysteamine was essentially the same as for the other thiols used in this study at either cell density.

Twenty-four h prior to drug exposure at different temperatures exponentially growing cells were trypanized (0.25% trypsin not supplemented with EDTA for 2 min, 37°C) and counted. Dilutions of known cell numbers were inoculated in T-25 flasks (Falcon Plastics) containing 4.5 ml of fresh medium (total volume). The flasks were placed in a 37°C incubator containing 5% CO2 and air until treatment. Cysteamine (12.5 to 100 μl) was added directly to the warm medium (pH 7.4). The pH remained constant for at least 2 h as measured after the time of drug and/or heat treatment. After 2 h drug exposure at 37°C, the medium was removed, and cells were washed twice with warmed PBS and then trypsinized. For experiments performed at 5°C, the closed flasks were placed in a refrigerator and maintained for 20 min at this temperature before adding the drug. After 2 h total drug exposure time at 5°C, cysteamine containing medium was removed, and cells were also washed and trypsinized. In studies involving hyperthermia, the cells were treated with cysteamine for 30 min at 37°C before heat treatment at 44°C. The flasks were placed horizontally in a circulating water bath (Umwälz-Thermostat W45/EB; Haake AG, Berlin, West Germany) at 44 ± 0.05°C (range) and then returned to a 37°C incubator. In some experiments, cells were heated for various periods of time at 44°C and then immediately washed and trypsinized. If not indicated otherwise, the total time of exposure to cysteamine in all experiments was 2 h including the time of heat treatment.

The influence of thiols upon GSH content or upon clonogenic cell
survival at 37°C were further examined by the addition of catalase (50 
µM/ml; Sigma Chemical Co.). Catalase was added to the culture medium 
at 37°C in a small volume (50 µl) 10 min before the cells were exposed 
to drug treatment.

Some studies involved pretreatment of cells with BSO (5 µM) for 15 h 
at 37°C prior to thiol exposure. Six h after the cells were plated, BSO 
(50 µM) was added to the warm medium. Following the pretreatment 
with BSO for 15 h, cells were exposed to the thiol compound for another 2 h 
in the presence of BSO. Then the medium was removed and the cells 
were washed and trypsinized. From our experience using BSO, we 
followed this procedure (5 µM BSO, 15 h) to avoid cytotoxic effects of 
the compound when used at high concentrations in CHO cells (12). For 
comparison, the effect of BSO without pretreatment of cells was also 
investigated. In these experiments, BSO was added at 37°C immediately 
before thiol treatment and was present only during the 2-h thiol exposure.

**GSH Determination.** Experiments designed to measure total intracel-

lar GSH were initiated by inoculating 10⁴ cells/flask in two replicate 
T-25 flasks containing 4.5 ml of fresh medium (total volume). The flasks 
were placed in a 37°C incubator containing 5% CO₂ and air 24 h prior 
to drug treatment. After thiol exposure at different temperatures (see 
above), the medium was removed, and cells were washed twice with 
warmed PBS and then trypsinized (0.25% for 2 min). At the time of GSH 
measurement, 96% of such treated cells were metabolically viable as 
assessed by trypan blue exclusion. After dilution in cold PBS (5 ml), the 
cells were counted and centrifuged at 4°C. The pellets were resuspended 
in 0.3 ml of 0.04 M EDTA and 0.6 ml of 7.5% trichloroacetic acid, 
vortexed, and centrifuged again at 4°C. The cold supernatant was 
removed and assayed for total GSH by the GSH reductase procedure 
(13). Protein determinations were made by the method of Lowry ef al. 
(14). The GSH content of control cells throughout the study was (n = 8) 
24.2 ± 6.1 (SE) nmol/mg protein or 3.3 ± 1.1 nmol/10⁶ cells. Although 
the GSH content of control cells varied from experiment to experiment, 
the relative changes in GSH induced by thiols and/or heat treatment 
were highly reproducible for each experiment. The control value of cellular 
GSH was not affected by the washing procedure with PBS and also in 
the same range for cells which were either trypsinized or scraped off, 
using a rubber policeman prior to GSH determination. Determination of 
GSH content of CHO cells after pretreatment with BSO (5 µM) for 15 h 
at 37°C showed a reduction of GSH (4.5 ± 1.0 nmol/mg protein or 0.5 
± 0.2 nmol/10⁶ cells) to about 20% of the control value under these 
conditions.

**Clonogenic Cell Survival.** Survival experiments were initiated by 
inoculating the appropriate number of cells (10⁴–10⁵ cells/flask) in four 
replicate T-25 flasks containing 4.5 ml of fresh medium (total volume) 
necessary to yield 50 to 100 colonies. The flasks were placed in a 37°C 
incubator containing 5% CO₂ and air 24 h prior to drug treatment. After 
2 h total thiol exposure time at 37°C (see above), the medium was 
removed, and cells were washed twice with warmed PBS (4 ml) and 
then covered with complete medium. The cells were incubated for 7 to 
9 days for colony development. After incubation, the colonies were rinsed 
with 0.9% NaCl solution, fixed, and stained with ammonium oxalate 2% 
crystal violet in 20% ethanol. Following colony formation, the fraction 
of treated cells giving rise to colonies (≥50 cells/collection) was normalized to 
the fraction of control cells giving rise to colonies (plating efficiency). 
Plating efficiency of control cells treated under these conditions without 
drug was identical to that observed for cells maintained at 37°C. Also 
plating efficiency was not affected when cells were kept in a cysteamine-
free medium for 2 h at 37°C. The surviving fraction was calculated after 
correction for cellular multiplicity (approximately 1.9), which was deter-
mined at the time of heat and/or drug treatment. For comparison, the 
incidence of cell density during thiol treatment at 37°C and 44°C was 
also examined. After thiol exposure at high density (≥10⁶ cells/flask), the 
medium was removed, and the cells were washed, trypsinized, and 
plated in four replicate T-25 flasks (10⁵–10⁶ cells/flask) for determination of 
clonogenic cell survival.

**Chemicals.** Cysteamine hydrochloride (β-mercaptoethylamine), cyste-
ine hydrochloride, N-acetylcysteine hydrochloride, and dithiothreitol 
were all obtained from Sigma. OTZ was kindly supplied by J. Biaglow, 
Cleveland, OH. The drug was originally synthesized by A. Russo (National 
Cancer Institute, Bethesda, MD) using the procedure as described by 
Kaneko et al. (15). All thiols were stored at 5°C in a desiccator. Stock 
solutions were freshly prepared by dissolving the chemicals in cold 
Dulbecco’s phosphate buffered saline solution, which was previously 
gassed with N₂ for 30 min. The pH was adjusted to 7.3–7.4. The stock 
solutions were sterilized by filtering through a 0.2-µm Millipore membrane 
and kept on ice until use (1 h). Catalase from bovine liver (17,600 units/ 
mg) was obtained from Sigma. BSO was obtained from Chemalog, South 
Plainfields, NY. Stock solutions of these drugs were freshly prepared in 
Dulbecco’s solution, sterilized as described above, and kept on ice.

**RESULTS**

Cysteamine induced an elevation of total GSH content in CHO 
cells (Chart 1A). At 0.4 mm cysteamine, a rather linear increase of 
GSH over time could be observed at 37°C. In repeated sets of 
experiments, the maximum content of GSH after a 2-h exposure 
to cysteamine was about 55 ± 2.1 nmol/mg protein (2–4-fold 
increase as compared to control values) and never exceeded 
this level by further incubation (3 h) with the drug (data not 
shown). No significant increase of GSH was found when cells 
were kept at 5°C during drug exposure. The elevation of GSH 
was more rapid when cells were exposed to an additional heat 
treatment (44°C, 30 min) during drug incubation at 37°C (Chart 
1B). The increase in GSH by heat treatment alone was found to 
be much less pronounced as compared to the combined heat 
and drug treatment.

Further analysis of the influence of heat (44°C) upon the 
cysteamine induced GSH elevation is shown in Chart 2. After a 30-
in incubation at 37°C, heat treatment was given for 
indicated lengths of time (minutes at 44°C) in the presence of 
cysteamine (0.4 mm). As seen in Chart 2, the initial rapid increase 
in GSH after 5–10 min heat treatment is followed by a rather 
slow increase which is most similar to a saturation effect.

In one set of experiments, we further addressed the question 
of whether or not the cysteamine induced increase of GSH is 
due to new biosynthesis of GSH. Cells were pretreated with 5
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Chart 2. GSH (nmol/mg protein) as a function of time for CHO cells (=10⁵ cells/flask) heated at 44°C after a pretreatment (30 min) at 37°C in the presence of 0.4 mM cysteamine. Points, replicate experiments; bars, SE.

Chart 3. GSH (nmol/mg protein) as a function of different concentrations of cysteamine (0-2.0 mM) for CHO cells (10⁵ cells/flask) exposed to the drug for 2 h at 37°C in complete McCoy’s Medium 5A, in cystine-free medium, or after treatment of cells with BSO (5 μM) for 15 h at 37°C in complete medium in the presence of BSO. For experimental details, see “Materials and Methods”). Points, replicate experiments; bars, SE.

μM BSO for 15 h at 37°C followed by exposure to cysteamine (0-2.0 mM) for 2 h at 37°C in the presence of BSO. Also in another set of experiments, cells were kept in a cystine-free medium for 2 h during drug exposure (0-2.0 mM) and the GSH content was determined immediately thereafter. The results of these experiments are shown in Chart 3. Dependent on different concentrations of cysteamine, the induced increase of cellular GSH at 37°C was most pronounced at low concentrations (0.1-0.8 mM) of the drug when cells were incubated in complete McCoy’s Medium 5A. This elevation in GSH content was not observed when cells were kept in a cystine-free medium during drug exposure. In cells pretreated with 5 μM BSO for 15 h at 37°C, no increase of GSH was found after exposure to different concentrations of cysteamine when BSO was further present during the 2-h drug treatment. BSO pretreatment of cells under these conditions reduced the GSH content to about 20% of the control value (see also “Materials and Methods”). Also no increase in GSH by cysteamine was observed in experiments in which BSO was added immediately after cysteamine treatment and was present only during the drug treatment.

The decay of the increased level in GSH after thiol treatment in CHO cells was followed over time, and the results for cysteamine (0.4 mM) are shown in Chart 4. A rather linear decrease in GSH could be observed for 8 h when cells were kept in complete McCoy’s Medium 5A at 37°C after a 2-h drug treatment at the same temperature. When an additional heat treatment (44°C, 30 min) was given during cysteamine exposure, the decay in GSH was less rapid, but within 22 h GSH values decreased in both cases to near control levels. From 2 to 4 h after heat treatment alone (44°C, 30 min), only a slight increase in GSH could be observed which never approached the level of GSH induced by cysteamine at 37°C or by cysteamine combined with heat (44°C, 30 min).

The most pronounced increase in GSH content was found at low concentrations of cysteamine (0.2-1.2 mM), which parallels the concentration range for the cytotoxic effects of this drug (8, 16). For comparison, we also determined the influence of other thiols like cysteamine upon GSH levels in CHO cells at comparable toxic concentrations known to generate activated oxygen species in the medium (10, 17). Chart 5A shows the data for clonogenic cell survival of CHO cells exposed to different concentrations of cysteamine, cysteine, N-acetylcysteine, and dithiothreitol for 2 h at 37°C. Similar to our previous results with cysteamine (8), we found a cytotoxic effect for the other thiols at low concentrations only, which was most pronounced for cysteamine followed by cysteine, N-acetylcysteine, and dithio-
dithiothreitol. The maximum reduction of clonogenic cell survival by these thiols was observed in the concentration range of 0.4–1.0 mM and comparable at 0.8 mM for all thiols which reduced the surviving fraction to about 5 to 15%. Chart 58 shows the results of clonogenic cell survival of CHO cells treated with the different thiols (0.8 mM) for indicated lengths of time at 37°C. Again the surviving fraction was similarly reduced for cysteamine, cysteine, N-acetylcysteine, and dithiothreitol over time but only if cells were plated at low densities. It should be emphasized at this point that the surviving fraction of cells treated with thiols at 37°C was not affected when cells were plated at high cell density (105–106 cells/flask) or when catalase (50 µg/ml) was added prior to thiol treatment. The influence of the different thiols on GSH content at 37°C and the results with cysteine, N-acetylcysteine, and dithiothreitol compared to the results obtained with cysteamine are shown in Table 1. After thiol exposure (0.8 mM) for 2 h at 37°C, the GSH content of cells was markedly increased. The elevation in GSH was most pronounced after exposure to dithiothreitol, a bifunctional compound with regard to sulfhydryl groups. Again in the presence of BSO, no increase of GSH was found after exposure to any thiol investigated either when cells were pretreated with BSO or when BSO was present during thiol treatment only. The elevation of GSH after any thiol treatment was not modified by catalase (50 µg/ml) added immediately before drug treatment. Interestingly when cells were kept in a cystine-free medium during drug treatment, elevated GSH levels could be observed in the case of cysteamine and dithiothreitol, but no increase in GSH was found after cysteamine or N-acetylcysteine treatment.

DISCUSSION

Several studies have shown that intracellular GSH is of major importance in protecting cells against damage by toxic compounds (3), radiation (18), and heat (19). GSH was also found to play an important role in the detoxification of activated oxygen (1, 4). It has been shown previously that thermosensitization of cells occurred by exposure to different concentrations of the thiol compound cysteamine (7, 8). Activated oxygen species like superoxide (O2•−) and hydrogen peroxide (H2O2), which are generated during antioxidation of thiols in the presence of oxygen (10, 17), are involved in the mechanism(s) of this effect (8, 9). During thiol exposure at elevated temperatures GSH may be necessary to balance the enhanced rate of H2O2 production (10) or lipid peroxidation (20) and to scavenge hydroxyl radical intermediates which might be subsequently formed under the combined oxidative and thermal stress (9).

In the present study, we found a marked increase in intracellular GSH in CHO cells after incubation in media supplemented with cysteamine. The content in GSH approximately doubled...
Our results suggest that the cysteamine induced increase in GSH results from de novo synthesis of GSH rather than from cleavage of mixed disulfides because the GSH elevation can be completely blocked by BSO treatment. BSO acts as an inhibitor of γ-glutamylcysteine synthetase, the first enzymatic step of GSH biosynthesis (23). During preparation of this manuscript, we were aware of a recent work done by Russo and Mitchell (24), who studied the radiation response of cells after elevation of cellular GSH by several compounds including cysteamine at radioprotective concentrations (10 μM). Similar to our findings, the authors reported an increase in GSH due to cysteamine and that the effect was blocked by BSO.

The present studies (using a cystine-free medium) also suggest that cysteamine acts as a delivery system for cysteine from the medium into the cells. According to this idea, no increase in cellular GSH would be expected in a cystine-free medium. This, however, was observed in studies with cysteamine and N-acetylcysteine, where the elevation in GSH synthesis of both thiols was dependent on cystine in the medium. A net increase of GSH in isolated rat hepatocytes after cysteine exposure has been reported by Beatty and Reed (25). Similar to our results, they found that the elevation in GSH induced by cysteine was independent of cystine present in the medium at the time of starting the experiment. The ability of diithiothreitol to provide cells with cysteine even when experiments are performed in a cystine-free medium might be due to the interaction of this bifunctional sulfhydryl compound (containing two sulfhydryl groups) with serum albumin which is present abundantly in the culture medium. Cysteine, which masks partially the reactive thiol groups of albumin (26), could be released by diithiothreitol via thiol-disulfide exchange reactions as shown for other thiols (27). In support of this idea, Ishii et al. (28), studying growth promotion of L1210 cell lines, have shown that cells obtained a high capacity to utilize cystine in the medium in the presence of 2-mercaptopetoanaloxol by formation of a mixed disulfide. The same authors also reported an increase in the GSH content of L1210 cells after incubation with 2-mercaptopetoanaloxol in the presence of cystine (29). The ability of cysteamine to promote cysteine transport from cystinotic skin fibroblasts has also been reported (30).

We conclude from these data that our results with cysteamine and related thiols fit in the proposed action of such compounds to promote cysteine uptake into the cells. Intracellular cysteine indirectly provided by these compounds seems to be essential for GSH synthesis in CHO cells. For comparison, we also used OTZ in these studies, a compound bearing a thiol group as part of a ring structure that is enzymatically opened and then directly serves as an intracellular pool for cysteine itself (31). The difference in the observed induction of GSH biosynthesis at equimolar concentrations of thiols compared with OTZ is most probably explained by the different mechanisms of GSH elevation induced by OTZ and thiols.

The uptake of cysteine through the cell membrane might be a critical factor in the temperature dependence of GSH elevation. Also the kinetics for the increase at 44°C was different from that at 37°C (Charts 1 and 2). Recently we have completed a study designed to determine the rate of cysteine transport induced by thiols at different temperatures. The results strongly support our proposed model of transport and utilization of labeled cysteine for GSH biosynthesis. In our studies, the observed decay of elevated GSH levels was rapid and was not remarkably modified by an additional heat exposure given during the initial drug treatment. A more pronounced elevation of GSH induced by heat alone has been reported using different experimental conditions (19). Also the increased GSH content of cells kept at 37°C after heat has remained at this level for at least 9 h (19). Further studies are in progress in our laboratory to determine the metabolic utilization of increased GSH after thiol exposure or heat treatment which might be different.

In the present paper, the elevation in GSH synthesis by thiols parallels the observed cytotoxicity of these compounds (Chart 5). The similar temperature dependence (8) and the maximum of both effects at low thiol concentrations suggest a possible synergism at the molecular level between the rapid elevation in GSH synthesis and the generation of activated oxygen species during thiol exposure. However, in the presence of catalase, which completely blocks the cytotoxic effects of thiols (8, 9), the increase in GSH is not affected (see Table 1). If synergism exists, the interaction of activated oxygen species with increased levels of GSH during thiol incubation should produce additional toxic product(s) that further react with structural and/or metabolic targets of cells. The oxidation of cysteine in the presence of oxygen forms stable products via free radical intermediates (e.g., thyl radical) (32). Through their reaction with molecular oxygen or hydrogen peroxide, thyl radicals can lead to the production of oxygen containing derivatives (32). Among the most interesting possibilities for free radical formation is the thiol group of GSH. Also production of hydroxyl radicals (OH), the most powerful one electron oxidant in biological systems and reactive with, e.g., DNA or membranes, has been shown to be enhanced by GSH and other thiols at low concentrations in the presence of hydrogen peroxide (33). At high thiol concentrations, scavenging of OH radicals could be observed (33). The concentration dependence of cytotoxic effects of thiols (see Chart 5A) and also of the thermosensitizing effects of cysteamine (8) could be based on the interaction of elevated cellular GSH with activated oxygen species generated at low concentrations only. This interaction could cause significant perturbation of the intracellular GSH system as shown in detail for the herbicide parquat in perfused rat liver (34). Why the cytotoxic effects mediated by thiols are completely dependent on cell density as observed in the present study remains unexplained.

In conclusion it seems possible that the generation of radicals during oxidative and/or thermal stress, which further react with GSH, causes significant perturbation of the intracellular GSH system. The formation of glutathione disulfide or of protein mixed disulfides following thiol treatment at low concentrations could be an important step in the expression of cytotoxicity and thermosensitization by these drugs. Further studies are in prog-
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ress to measure in more detail such alterations in the cellular GSH system during oxidative and thermal stress.

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