ABSTRACT

HA-1 Chinese hamster fibroblasts and two heat-resistant variants, designated 2242 and 3012, have been investigated to determine the role that glutathione (GSH) plays in intrinsic cellular resistance to heat and in the development of thermotolerance. The constitutive levels of GSH did not correlate with intrinsic heat sensitivities, but depletion of GSH sensitized all three cell lines to thermal stress. After heating (43.5°C/2 h), surviving fractions were $1 \times 10^{-4}$, $1 \times 10^{-3}$, and $8 \times 10^{-3}$ for HA-1, 2242, and 3012 cells, respectively. Depletion of cellular GSH with l-buthionine-$S,R$-sulfoximine to less than 10% of control values sensitized such that the thermal responses of these three cell lines were nearly indistinguishable at 43.5°C. Surviving fractions were $2 \times 10^{-4}$, $1 \times 10^{-3}$, and $1 \times 10^{-4}$ for l-buthionine-$S,R$-sulfoximine-treated HA-1, 2242, and 3012 cells, respectively, following heating at 43.5°C for 2 h.

The development of thermotolerance in HA-1 cells following heat shock (45°C/15 min) was unaffected by the inhibition of GSH synthesis. On the other hand, when GSH levels were maintained at extremely low levels, the development of thermotolerance was inhibited. In addition, following heat shock, cellular GSH was decreased and remained below control levels during the development of thermotolerance.

INTRODUCTION

The tripeptide thiol GSH has been regarded as having an important role in protection of cells from environmental insults, including ionizing radiation, toxic chemicals, and heat (1). Because of this, sulfhydryl reagents, such as diethyl maleate or N-ethyl maleimide, or GSH oxidizing agents, such as diamide, have been used to deplete cellular GSH in vitro in order to study the effects of cancer treatment modalities in experimental systems. More recently, an inhibitor of glutathione synthesis, BSO, has been used to deplete cellular GSH because it is regarded to be less toxic than other agents (2).

In several reports, Mitchell and coworkers have found that depletion of GSH resulted in thermosensitization of cultured hamster fibroblasts (3–5). They also found that heat shock resulted in an increase in cellular GSH associated with production of heat shock proteins and thermotolerance. Inhibition of GSH synthesis following heat shock resulted in reductions in heat shock protein synthesis and in the degree of thermotolerance developed.

The purpose of the present study was to determine the role that cellular GSH may play in cellular sensitivity to thermal stress and in the development of thermotolerance. Of particular interest was the role that GSH played in the intrinsic thermostensitivity of two stable heat-resistant variants of Chinese hamster fibroblasts and what effect depletion of cellular GSH would have on this intrinsic resistance to hyperthermic killing. In addition, we have further investigated the role that GSH synthesis plays in the development of thermotolerance following heat shock.

MATERIALS AND METHODS

Cell Lines. Chinese hamster HA-1 fibroblasts were grown as monolayer culture, in minimum essential medium with 15% fetal calf serum and antibiotics. The heat-resistant cell lines were derived from HA-1 as previously described (6). The plating efficiency was 70–90% for all cell lines.

For experiments, cells were seeded into 60-mm Petri dishes and grown for approximately 48 h to a density of $5 \times 10^{4}$ cells/cm² before heating. After heat treatments, cells were trypsinized (0.05% trypsin-0.02% EDTA, 37°C, 3–5 min), diluted, and replated for colony formation and determination of surviving fraction. An aliquot of cells was also reserved for determination of cellular GSH.

Water Bath Heating. Cells were heated in a specially constructed water bath incubator with precise control of both temperature and CO₂ (6). Growth medium was replaced immediately before heating.

GSH Depletion and Measurement. BSO, an inhibitor of $\gamma$-glutamyl-cysteine synthetase (2), was used to inhibit GSH synthesis and deplete cells of GSH. BSO was added to the culture medium to a final concentration of 50 $\mu M$. This concentration decreased cellular GSH with a half-time of about 2 h. The cellular GSH was decreased to <10% within 6–7 h. In these experiments, cells were exposed to BSO for no longer than 18 h. This treatment alone did not affect cell growth or viability. BSO was always removed prior to heat treatments.

GSH was measured by the method of Tietze (7). Briefly, cells were trypsinized, centrifuged at 4°C, and resuspended in cold 0.6% sulfosalicylic acid. The acid-soluble material was then assayed for GSH by measuring the change in color of DTNB at 412 nm in the presence of glutathione reductase and NADPH.

RESULTS

Effect of GSH Depletion on Thermal Sensitivity. Survival of HA-1, 3012, and 2242 cells following heating at 43.5°C is shown in Fig. 1. 3012 and 2242 cells characteristically showed greater heat resistance than the parent HA-1 line. For example, at a heating time of 120 min, SFs were $6.5 \times 10^{-4}$, $7 \times 10^{-3}$, and $3.5 \times 10^{-2}$ for HA-1, 3012, and 2242 cells, respectively.

When these cells were exposed to 50 $\mu M$ BSO for 12–14 h, which reduced cellular GSH to <10% of control levels, and then heated at 43.5°C, all three cell lines were sensitized to killing by 43.5°C heat (Fig. 1). The rates of GSH depletion by BSO were similar in the three cell lines (t½ ~ 1.7–2.0 h). The effect of GSH depletion was different in each cell line, having the greatest relative sensitizing effect in the most resistant 2242 cells. The survival curves resulting from heating the three cell lines following GSH depletion were almost indistinguishable from one another.

The intrinsic heat sensitivities of HA-1 cells and the heat-resistant variants did not correlate with constitutive cellular GSH. In fact, the measured GSH contents of the heat-resistant 2242 and 3012 were slightly lower than that of HA-1 cells (Table 1).

Time Course of GSH Depletion and Heat Sensitization by BSO. When HA-1 or 2242 cells were exposed to 50 $\mu M$ BSO for increasing times and then heated at 43.5°C for 2 h, a time-
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Effects of Inhibition of GSH Synthesis on Development of Thermotolerance. When HA-1 cells were given an initial heat dose of 45°C/15 min, followed by a period of time at 37°C, and then given a second heat dose of 43.5°C/90 min, the resulting survival increased as the time between heatings was increased (Fig. 3a). This phenomenon, observed in most mammalian cell lines, is usually referred to as the development of thermotolerance. In order to investigate the role of GSH synthesis in this process, we performed this split-dose heating experiment in several ways as follows.

BSO Present Only between Heat Treatments. When cells were given the initial heat treatment, then incubated for up to 6 h in the presence of 50 µM BSO before the second heating, the pattern of increasing survival was similar to that observed for cells never exposed to BSO (Fig. 3a). During the time that thermotolerance was developing, the GSH content of these cells was decreasing due to the inhibition of GSH synthesis by BSO (Fig. 3b). By 6 h the cell survivals resulting from the combined heat treatment were identical in the two treatment groups (Fig. 3a) despite a nearly 10-fold difference in cellular GSH (Fig. 3b).

BSO Present Only before the Initial Heat Treatment. When HA-1 cells were treated with BSO for 12 h prior to the first heating, a significant heat sensitization was observed (Fig. 3a). GSH-depleted cells given the two heat treatments in succession at 37°C with no interval in between demonstrated 10-fold lower survival than GSH-proficient cells. As the interval between heatings was extended (in BSO-free conditions), these cells demonstrated a rapid recovery from heat sensitization, and by 6 h, they survived the combined heat treatment as well as cells that were never exposed to BSO.

The GSH level in these cells was initially very low (less than 10% of control; Fig. 3b), but during the period at 37°C between heatings in BSO-free medium, GSH increased rapidly (t½ = 1.7 h) and had reached approximately 50% of control by 6 h. This rate of regeneration of cellular GSH was similar for heated and unheated cells washed free of 50 µM BSO (data not shown).

dependent heat sensitization was observed (Fig. 2). As expected from the above survival curves (Fig. 1), 2242 cells were initially much more resistant than HA-1 cells. 2242 cells showed greater sensitization with time, and by 6 h they were nearly as sensitive as HA-1. Further GSH depletion by exposure to BSO for 8 h sensitized HA-1 and 2242 to the maximum sensitivity observed, and sensitization was such that survival of the two cell lines was similar following heating at 43.5°C for 2 h.

Table 1 Glutathione content and heat sensitivities of HA-1 and two heat-resistant variants

<table>
<thead>
<tr>
<th></th>
<th>HA-1</th>
<th>3012</th>
<th>2242</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GSH] (fmol/cell)*</td>
<td>5.3-7.3</td>
<td>3.7-4.8</td>
<td>3.2-9.9 x 10^-4</td>
</tr>
<tr>
<td>SF (43.5°C/2 h)*</td>
<td>4.2-4.7</td>
<td>4.5-9.9 x 10^-3</td>
<td>2.6 x 10^-2</td>
</tr>
</tbody>
</table>

* Range of 3–5 measurements.

Ranges of 2–4 separate experiments.

Fig. 3. a, survival of HA-1 cells following split dose heating. A first dose of 45°C/15 min was followed by an interval (0–6 h) at 37°C and a second heating at 43.5°C/90 min. Symbols: O, no BSO treatment; •, 50 µM BSO present only during interval between heatings; □, 50 µM BSO present only for 12 h before first heating; ■, 50 µM BSO present for 12 h before first heating and during interval between heatings. Arrows indicate SFs following single heat treatments. A, SF expected by multiplying that found following 45°C/15 min by that following 43.5°C/90 min; B, same as A for BSO-pretreated cells. b, glutathione levels for cells treated as in a. GSH was measured before the second heating. (I.e., all cells were heated at 45°C/15 min at time 0. Symbols: same as in a. Dashed line, control level of GSH for HA-1 cells. The paired results from one of two replicate experiments are shown.

Fig. 2. Time course of heat sensitization of HA-1 and 2242 cells by GSH depletion. Cells were exposed to 50 µM BSO for the time shown. Cultures were washed free of BSO, then heated for 2 h at 43.5°C. Points, results of one of three separate experiments.
BSO Present before the First Heat Treatment and between Heat Treatments. When cells treated as above (i.e., 50 μM BSO, 12 h) were also incubated in BSO-containing medium during the interval between heat treatments, GSH levels were maintained at extremely low levels (i.e., < 10% of control; Fig. 3b). Even under these conditions some increase in survival was observed with increasing time between heatings (Fig. 3a). However, although cells treated in this manner showed over 100-fold increase in survival over the first 4-h period, no further increase in survival was observed. The final SF (i.e., 6 h) was approximately 1% of that attained by the other treatment groups.

Effect of Heat Shock on Intracellular GSH in HA-1 Cells. Intracellular GSH was measured in HA-1 cells following heating at either 45°C for 15 min or 43.5°C for 45 min. Each treatment reduced survival to approximately 0.40. Intracellular GSH fell below control levels immediately following heat shock and remained at levels significantly below control for up to 6 h after heating, the longest time tested (Fig. 4). The results of many GSH determinations before and after various BSO and heat treatments have consistently shown that, in all cases, GSH was lower following heating (data not shown).

DISCUSSION

Our results indicate that depletion of cellular GSH by BSO can effectively sensitize cells to killing by 43.5°C hyperthermia. This is in agreement with the initial findings of Mitchell et al. (4). In our studies thermal sensitization by GSH depletion was shown to be effective in heat-resistant cells as well as in the parent cell line. This ability to overcome heat resistance, which is not associated with elevated levels of GSH, is particularly intriguing. The results suggest that, while the intrinsic heat resistance of 3012 and 2242 cells was not directly due to cellular GSH, resistance was no longer expressed when GSH levels were extremely low.

Following heating at 43.5°C or 45°C, cellular GSH levels were lower in HA-1 cells compared to unheated cells (Fig. 4). In addition, development of thermotolerance in HA-1 cells was not associated with an increase in cellular GSH (Fig. 3, a and b). Thermotolerance developed equally well under conditions of steady-state GSH, decreasing GSH, or recovering GSH following depletion by BSO (Fig. 3, a and b). Only when very low levels of GSH were attained and maintained throughout the induction and development periods was the degree of thermotolerance development diminished. These results may be explained based on the involvement of enzymes that cycle GSH (e.g., GSH reductase, GSH peroxidase), and that may be affected only by depletion of cellular GSH to extremely low levels.

The survival levels labeled “A” and “B” in Fig. 3a indicate the survivals expected following the two heat treatments based on the multiplication of the survivals obtained by the individual treatments. These survival levels (A and B) are what would be obtained if there were no interaction between the two heat treatments, there had been full recovery of the survival curve shoulder, and if there were no significant cell cycle redistribution following the first heating. All treatment groups demonstrated survivals greater than these levels when the interval between heatings was 6 h, except the group that was maintained in BSO and at low GSH concentrations throughout the experiment. This latter group approached survival level B, suggesting that some recovery from damage caused by the first heating had occurred in the interval between heatings, but that no thermotolerance was observed.

Our results differ in two respects from those of others (3–5, 8, 9). (a) Heat shock did not induce an increase in cellular GSH in our studies. (b) The development of thermotolerance was not dependent on the new synthesis of GSH. However, the findings of Mitchell et al. (3–5) that GSH depletion leads to thermal sensitization at temperatures around 43°C and that cells severely depleted of GSH for a prolonged period are compromised in their ability to develop thermotolerance are confirmed by our results. The discrepancies in results may arise from the use of different cell lines, which may respond differently to heat treatments (10). Although the GSH contents of our cells and those used by Mitchell et al. are similar, it remains possible that levels of GSH-utilizing enzymes or utilization of various metabolic pathways involving cycles of oxidation and reduction may be different from cell line to cell line. These differences and their importance relative to cellular thermal sensitivity remain largely unknown.

In summary, our results indicate that: (a) the intrinsic heat sensitivities of HA-1 cells and their heat-resistant variants 3012 and 2422 are not correlated to the constitutive cellular GSH concentration; (b) cells could be sensitized to heat treatment by GSH depletion with BSO. This sensitized all three cell lines such that, following GSH depletion, 3012 and 2422 cells were no longer more heat resistant than HA-1; (c) development of thermotolerance was not correlated with an increase in cellular GSH; and (d) thermotolerance could be expressed in the presence of BSO, an inhibitor of GSH synthesis.

These results suggest that depletion of GSH could increase the effectiveness of hyperthermic killing of tumor cells. The combination of GSH depletion with hyperthermic killing would be expected to be effective on the entire tumor cell population, including the heat-resistant subpopulations. Thus, the combination offers advantages over combining GSH depletion with X-radiation or chemotherapy: GSH depletion combined with these modalities would either act on only a small population of tumor cells (i.e., hypoxic cells in the case of X-radiation) or would not allow for localized treatment, thereby putting normal tissues at increased risk (in the case of chemotherapy). In the case of localized hyperthermia, however, deleterious effects of sensitization of normal tissues to heat may be avoided in some sites.

Glutathione depletion could sensitize all three cell lines studied to killing by heat. On the other hand, cells that were resistant to heat through development of thermotolerance were not sensitized by GSH depletion. This suggests that cells may become heat resistant by various mechanisms, some that may be overridden by GSH depletion (e.g., stable heat-resistant...
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2242, 3012 cells) and others that are not affected by cellular GSH (e.g., transiently thermotolerant HA-1 cells). The mechanisms by which cells become heat resistant and the mechanism of heat sensitization by GSH depletion remain unclear at this time.

REFERENCES

Cellular Glutathione, Thermal Sensitivity, and Thermotolerance in Chinese Hamster Fibroblasts and Their Heat-resistant Variants

Dennis C. Shrieve, Gloria C. Li, Anna Astromoff, et al.


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