Glutathione Elevation during Thermotolerance Induction and Thermosensitization by Glutathione Depletion

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ABSTRACT

Chinese hamster V79 cells were made thermotolerant by either continuous heating at 42.5°C or by fractionated 43°C exposures with interfraction incubation at 37°C. For both methods of thermotolerance induction, elevations in cellular glutathione (GSH) were observed. Additionally, GSH was also shown to be elevated following a 1-hr exposure to 6% ethanol, which also induces thermotolerance. These elevations in cellular GSH preceded thermotolerance induction in regard to cell survival. To determine if a reduction in cellular GSH prior to or during heating at 42.5°C would influence thermotolerance, GSH levels were reduced by either pretreatment with diethylmaleate, an agent that binds GSH, or treatment during heating with buthionine sulfoximine, an agent that inhibits GSH synthesis. Both depleting protocols resulted in thermosensitization. These data suggest that GSH may be important in the early cellular response to thermal stress.

INTRODUCTION

The ability of cells to acquire transient resistance to thermal stress following a single heat treatment or prolonged continuous heating is well documented (10, 12, 34, 36, 38). Mechanisms proposed previously by which cells develop thermotolerance have evoked membrane fluidity changes (10), as well as the biosynthesis of stabilizing proteins, HSP.1 In Drosophila (28, 39), plants (21), and various mammalian cells (23, 41, 42), the adaptive response to thermal stress is mounted concomitantly with the production of HSP (23, 26). The mechanism by which these proteins provide survival benefit is not known (2, 23, 27). When cells acquire thermal resistance, not only are they resistant to subsequent heat exposure but also to selective cytotoxic chemical stress (25). Ethanol treatment has also been shown to induce thermotolerance, HSP, and resistance to cytotoxic drugs (25, 26).

In this study, we report yet another cellular response to thermal stress, that being the rapid elevation of GSH, a tripeptide involved in the maintenance of the cellular oxidation-reduction potential (8) and detoxification (19). These data suggest that cellular GSH levels may play an important role in the initial response of cells to hyperthermia stress.

MATERIALS AND METHODS

Cell Culture. Chinese hamster V79 cells were grown in Medium F12 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Biofluids, Inc., Rockville, Md.), penicillin G potassium (0.14 g/liter), and streptomycin sulfate (0.2 g/liter). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The general procedures for cell culture as described by Ham and Puck (15) were closely followed.

Cell Survival Analysis. Exponentially growing cells were removed from plastic stock flasks using a 0.9% NaCl solution containing 0.03% trypsin and EDTA (0.1 g/liter). The cells were counted using an Ethazone particle counter (Particle Data, Inc., Elmhurst, Ill.), and appropriate numbers of cells were inoculated into 25-sq cm plastic flasks containing 5 ml of medium and incubated 5 to 6 hr at 37°C before experimental procedures were started. Some studies involved treatment of the cells with 5 x 10^{-4} M DEM (Sigma Chemical Co., St. Louis, Mo.) or 10^{-2} M BSO (Chemalog, South Plainfields, N. Y.) prior to or during heating. Following the 5- to 6-hr incubation period, the medium was removed from the flasks and replaced with drug and medium or drug-free medium for control conditions. The cells were exposed to DEM or BSO for 2 hr at 37°C; the medium was then removed, the flasks were rinsed twice with a buffered 0.9% NaCl solution, and 5 ml of fresh medium were added. The 2-hr exposure to DEM or BSO did not affect the plating efficiency. The flasks were then gassed with 5% CO2 and 95% air, and the caps were sealed with paraffin wax to prevent leakage during water bath immersion. The pH of medium throughout all procedures was maintained between 7.2 and 7.4, as well as the biosynthesis of stabilizing proteins, HSP.1 In Drosophila (28, 39), plants (21), and various mammalian cells (23, 41, 42), the adaptive response to thermal stress is mounted concomitantly with the production of HSP (23, 26). The mechanism by which these proteins provide survival benefit is not known (2, 23, 27). When cells acquire thermal resistance, not only are they resistant to subsequent heat exposure but also to selective cytotoxic chemical stress (25). Ethanol treatment has also been shown to induce thermotolerance, HSP, and resistance to cytotoxic drugs (25, 26).

In this study, we report yet another cellular response to thermal stress, that being the rapid elevation of GSH, a tripeptide involved in the maintenance of the cellular oxidation-reduction potential (8) and detoxification (19). These data suggest that cellular GSH levels may play an important role in the initial response of cells to hyperthermia stress.

1 The abbreviations used are: HSP, heat shock proteins; GSH, glutathione; DEM, diethylmaleate; BSO, α-buthionine-S,R-sulfoximine.

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determinations were also made for cells exposed to ethanol. Cells were plated in 100-mm Petri dishes (about 10⁶ cells) and incubated 5 to 6 hr at 37°. The medium was then removed and replaced with medium containing 6% ethanol or ethanol-free medium. The cells were exposed 1 hr at 37° and rinsed twice with phosphate-buffered saline; then fresh medium was replaced, and the plates were returned to 37°. GSH determinations were made 5 hr after the ethanol treatment. Bars on charts represent the S.D. and are shown when larger than the symbols.

RESULTS

Thermotolerance was induced by 2 heating procedures. Shown in Chart 1A is the survival of V79 cells as a function of exposure time at 42.5°. Thermotolerance developed for this heating procedure after 2 to 5 hr of exposure as evidenced by a plateau in the survival response. This observation was consistent with that reported by others (10, 36). Chart 1B shows thermotolerance induction using a 1-hr 43° exposure followed by incubation at 37° and then reexposure to 43°. The 0-hr survival points represent the survival following the initial 1-hr 43° exposure, and the curve marked "0 hr" exhibits the survival for cells heated for various times at 43° without an interfraction interval at 37°. Survival decreased exponentially with time at 43°. Marked thermotolerance was observed as shown by the survival curve marked "5 hr", where cells following the initial 1-hr 43° exposure were incubated at 37° for 5 hr and then reexposed to various times at 43°. These data are similar to those reported by Li and Hahn (25).

The elevation in cellular GSH for V79 cells heated by both heating procedures used in Chart 1 is shown in Chart 2. GSH rapidly increased, reaching a plateau value of approximately 150% of control values by 1 hr for continuous heating at 42.5° (Chart 2A). The dashed area represents the mean ± S.D. of control GSH values for cells maintained at 37° during the 5-hr period. Control and heated samples in triplicate were assayed for each time point. After 5 hr of heating, the GSH values began to approach control values, and for one experiment (data not shown), the GSH content by 8 hr had returned to control values. The status of cellular GSH following a 1-hr exposure to 43° with subsequent incubation at 37° is shown in Chart 2B. The time indicated on the abscissa included the 1-hr 43° exposure. GSH also increased rapidly as a result of this heating protocol, reaching plateau values of 150 to 190% of control values. Again, the dashed area represents the mean ± S.D. of control GSH values for cells maintained at 37° during the 9-hr period.

To determine if a reduction in cellular GSH prior to heating at 42.5° would influence thermotolerance, cells were pretreated for 2 hr with DEM, an agent which binds the thiol group of GSH and thus removes GSH for further reactions. The cell survival data are shown in Chart 3. By pretreating cells with DEM, GSH levels were reduced to 38 and 49% of control values (closed symbols); however, this reduction in GSH had no effect on plating efficiency. Following this treatment, GSH gradually returns to control values in about 5 to 8 hr (data not shown). When DEM-pretreated cells were immediately heated at 42.5°, a pronounced enhancement of cell killing was ob-
served (Chart 3). Thus, DEM pretreatment greatly sensitized the cells to heat.

BSO has been reported to be a much more specific GSH-removing agent. Its major known mode of action is inhibition of γ-glutamylcysteine synthetase (9, 14). Therefore, BSO-treated cells have decreased ability to synthesize GSH. The results shown in Chart 4B represent survival of cells treated with BSO only during the respective heat exposures. GSH values (expressed as a percentage of control) for BSO-treated cells at 42.5°C for 1, 2, 3, 4, and 5 hr were 50, 25, 13, 8, and 6%, respectively. When BSO was present during heating, marked thermal sensitization occurred. As expected, if cells were pretreated with the synthesis inhibitor BSO, somewhat different findings were seen, as shown in Chart 4A. GSH levels were 22% of control values prior to heating, yet thermal sensitization was not as pronounced with BSO as with pretreatment with the irreversible binder DEM. It appears from this experiment that, although some sensitization occurred with BSO pretreatment, thermotolerance was induced, but at a somewhat lower survival level than for untreated cells.

Li and Hahn (25) have shown that thermotolerance can also be induced by treating cells with ethanol. In their experiments, 4 to 5 hr post ethanol treatment was the optimal timing for thermotolerance induction and Adriamycin resistance. In order to determine if cellular GSH was affected by ethanol treatment, several experiments were conducted where cells were exposed to 6% ethanol for 1 hr, rinsed, and then incubated at 37°C. Five hr after the ethanol exposure, GSH was determined for ethanol-treated cells and compared to control samples (Table 1). Clearly, cellular GSH values were elevated 5 hr post ethanol treatment to the same extent as that observed for thermally stressed cells.

DISCUSSION

It has long been known that, when cells are exposed to hyperthermic stress, thermotolerance may occur (10). Recently, it was observed that cells treated with heat or ethanol assumed resistance to further heating as well as chemical challenge by Adriamycin (25). It had been demonstrated previously in a variety of cell types that, with heating, there is an associated production of HSP (2). Subsequently, ethanol treatment was shown to produce HSP (26). The means by which, or in fact if, these proteins are integral to thermal resistance has not been answered. Part of the reason for the enigma is that the mechanism by which cells are killed by heat is not understood. Cell killing from heat has been proposed to result from protein denaturation (10), inhibition of protein synthesis (30, 32), membrane fluidity alterations (10), or, as suggested by a thermodynamic model, a series of complex rate-limiting reactions dependent upon particular temperature ranges (24).

Our interest in thermal tolerance stems from an interest in the mechanism by which cells become resistant to biochemical stress. Much work has been done on the mechanisms of cellular detoxification (19) and the means by which cells defend against ionizing radiation (33). GSH is known to react with electrophilic agents (19) as well as to play a pivotal role in the maintenance of a cellular oxidation-reduction state by shutting reducing equivalents. Also, GSH provides protection from free radicals and peroxides normally occurring in cells as a result of oxygen metabolism (8). The deleterious effects of peroxide production at the cellular level following exposure to ionizing radiation (33) and chemical agents (3, 7) are well documented. Kinetics theory and thermodynamics predict that with elevated temperature, the rate of production of peroxides and free radicals would increase, thereby necessitating, if survival were to be ensured, an increased requirement for reductive compounds such as GSH for detoxification. Therefore, it was of interest to determine if GSH is important in thermal response.

The purpose of this study was therefore 2-fold: (a) to measure the cellular GSH content during thermal stress; and (b) to examine if changing the cellular reducing capacity by altering GSH levels could influence thermosensitivity. Clearly, the data presented illustrate that, within the first hr of heating by either method, there is a rapid increase in cellular GSH, which remains elevated for a considerable length of time. Furthermore, when GSH levels are altered by DEM pretreatment or BSO treatment during heating, the cells are sensitized to thermal stress. DEM is an agent that binds GSH via 1,4 Michael addition of the thiol group catalyzed by GSH S-transferase (6, 35). However, it is not clear that this is the only mechanism by which DEM affects cells. For this reason, we also studied BSO, an agent whose only reported direct mode of action is competitive inhibition of γ-glutamylcysteine synthetase, a key enzyme in the biosynthesis of GSH (9, 14). Caution should always be used in ascribing a single cellular effect to a drug, in particular the indirect effects of GSH depletion (31, 37). Our
results indicate that depletion of GSH during heating with BSO, by an entirely different means from DEM, also sensitizes cells to heating at 42.5°C. Therefore, for cells heated at 42.5°C with BSO present, GSH was not synthesized as demonstrated by the progressive reduction in GSH concentration during heating. Thermal tolerance was not observed for BSO-treated cells, at least within the survival limits of the assay. Thermal tolerance might have occurred if cells had been heated for longer times and if survival could be accurately evaluated at survival levels below 10^-5. It is interesting to note that heating cells at 42.5°C following BSO pretreatment lowered the GSH levels to 22% of control values, yet it did not sensitize to the same degree as DEM pretreatment, which lowered the GSH to 38 and 49% of control values. This suggests that DEM may possibly be causing other effects at the cellular level, in addition to binding GSH, which enhances thermal sensitization. The hydrolysis product of DEM, maleic acid, as well as the thiol addition product, has been reported to cause alteration of glycolysis and also enzyme inhibition (43). The effects of various thiol-depleting agents on thermal sensitization, HSP synthesis, and amino acid transport (13) are presently under investigation in our laboratory.

In an attempt to examine if GSH elevation was associated only with thermal stress or, in fact, was a more generalized event that could be correlated with other forms of stress that also induce thermal tolerance, we studied the effects of ethanol treatment. Ethanol treatment is known to induce thermal tolerance (25). Our data show an elevation of GSH after ethanol treatment and support a relationship between elevated GSH and thermal resistance. It has also been demonstrated that approximately 5 hr post ethanol treatment, cells become more resistant to the cytotoxic agent Adriamycin (25). One of the proposed mechanisms of Adriamycin cytotoxicity includes the anion radical hydroquinone-mediated production of free radicals (4). If this mechanism is correct, then elevated cellular GSH, a free radical scavenger, produced as a result of ethanol treatment, would be anticipated in part to provide the cell with added protection from deleterious reactions associated with free radicals. In support of this view, Babson et al. (3) have shown recently that, when the GSH cycle is impaired, the cytotoxicity of Adriamycin is markedly increased. The observed thermal and chemical resistance may also be explained by the recent report that ethanol induced HSP (26), provided the assumption is made that these proteins confer protection to chemical and thermal vulnerable targets. Another possible mechanism of ethanol-induced resistance may be related to the changes postulated previously in membrane resulting from treatment with ethanol (25), which might affect Adriamycin transport. Preliminary experiments in our laboratory show that membrane transport of Adriamycin is not affected by ethanol pretreatment.3

When exogenous thiol compounds, such as cysteamine or cysteine, are present during hyperthermic exposure, they afford no protection from heat stress; in fact, these compounds promote enhancement of cell killing (20). These compounds have been demonstrated in numerous reports to provide protection from free radical production following ionizing radiation (33). That exogenous thiol compounds promote thermal sensitization is not clear. Both cysteamine and cysteine contain, in addition to a sulfhydryl group, an amino group. Several investigators have shown that amino-containing compounds potentiate hyperthermic killing (5, 11). In our laboratory, we have found that the reported thiol-mediated potentiation is markedly reduced if an acetate is used to block the amino group of cysteine.3 We are currently acquiring sulfhydryl-containing compounds, wherein the amino functional group is blocked and not subject to the action of amide-cleaving enzymes. These compounds should differentiate between the thermal sensitizing effect of an amino versus a thiol functional group.

Interestingly, glycerol, which affords radiation protection presumably by free radical scavenging (1), has been shown to protect cells exposed to heat (18). Henle et al. (16, 17) have suggested recently that polyol synthesis during thermotolerance induction affords thermal protection. Furthermore, Henle et al. postulate that these polyols would unquestionably alter the cellular oxidation-reduction state and hence cause perturbation in the GSH concentration.

Several plausible observations and speculations must be considered when our data are examined with regard to possible relationships between GSH and thermal sensitivity.

(a) GSH elevation may provide or be a reflection of reductive cycles in the cell that may confer transient thermal protection.

(b) GSH elevation may be necessary for the regulation of important thermal and chemoprotective macromolecular synthesis (e.g., HSP synthesis).

(c) GSH rapid elevation may be important in thermostolerance induction. Even though GSH levels plateau after 1 hr of heating and thermal tolerance develops after 2 to 5 hr of heating, it is possible that the initial increase in GSH concentration may initiate complex biochemical sequelae that require hr to be expressed fully.

(d) GSH depletion by 2 agents working by 2 different mechanisms can alter the thermal response. Aside from GSH depletion, these chemicals may also be effecting yet other cellular systems [e.g., protein synthesis (22, 29)] responsible for thermal sensitivity.

At the molecular level, it would be expected that, for elevated temperatures, there would be an increase in the entropy of highly ordered cellular processes; part of this disorganization might well be manifested by the production of toxic metabolites. We have demonstrated that GSH, a known cellular detoxifying agent (19), can be elevated by 2 different modalities—heat and chemical. Initial exposure to either modality may provide subsequent resistance to heat and/ or chemical stress. Modulation of GSH appears to correlate with thermal sensitivity. Future efforts in our laboratory will be directed to continued investigation of the role of GSH in thermal stress.

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REFERENCES


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GSH during Thermal Stress


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