Influence of Oxidative Stress Induced by Cysteamine upon the Induction and Development of Thermotolerance in Chinese Hamster Ovary Cells

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ABSTRACT

Chinese hamster ovary cells exposed to the sulfhydryl compound cysteamine combined with heat treatment at 44°C developed thermotolerance within 8 h. After initial treatment either with 15 min cysteamine (0.4 mM) at 37°C immediately followed by 15 min heat at 44°C or with 15 min cysteamine (0.4 mM) at 44°C, the magnitude of thermotolerance developed was identical. The ΔT of the subsequent 44°C heat survival curves increased by factors of 8.9 and 7.9, respectively. The kinetics of thermotolerance induction and the time to reach the maximum of thermotolerance expression after combined cysteamine treatment at 44°C for 15 min was found to be comparable to the effects of 44°C treatment alone for 30 min. The synergistic effect of cysteamine with the conditioning heat treatment at 44°C was blocked by catalase (50 μg/ml).

Following initial treatment with cysteamine at 37°C, cells became thermotolerant within 2 h. The ΔT of the survival curves for 44°C heat treatments increased with duration (t1 = min, 37°C) of the cysteamine (0.4 mM) exposure; e.g., the ΔT increased by factors of 1.5, 1.6, 2.2, and 2.6 for t1 = 30, 60, 90, and 120 min. The induction of thermotolerance by cysteamine at 37°C was completely blocked by the addition of catalase (50 μg/ml), present during the initial period of drug treatment.

Combined cysteamine and heat treatment at 44°C, but also cysteamine exposure at 37°C, enhanced synthesis of heat shock proteins. The data suggest that oxidative stress by cysteamine can be synergistic with the conditioning heat treatment at 44°C which induces thermotolerance. At 37°C, cysteamine itself induces thermotolerance and the enhanced synthesis of heat shock proteins under these conditions.

INTRODUCTION

Cells from various organisms have been shown to develop a transient thermal resistance after exposure to elevated temperatures (1, 2). This phenomenon is called thermotolerance and is recognized as a potentially important factor influencing the biological response to hyperthermia in vitro and in vivo. In the past years, the time and temperature dependence for the induction, development, and decay of thermotolerance has been studied extensively (3–5). The induction of thermotolerance in mammalian cells can be achieved in two different ways: (a) by continuous incubation at temperatures between 41.5°C and 42.5°C for periods in excess of 3 or 4 h, the cells become thermotolerant during the time of heat treatment (6, 7); (b) pretreatments at temperatures between 38°C and 42.5°C render the cells resistant to a second treatment at 43°C or higher (3, 8). By fractionated heat treatments at 43°C or higher, the cellular resistance to a second heat treatment is much more pronounced than by pretreatments below 42.5°C, but thermotolerance requires several h of incubation at 37°C to develop (1, 3, 9). Thermotolerance which developed during chronic heating or after acute heating seems to involve similar mechanisms. When cells became tolerant to chronic heating they have shown also to be tolerant to acute heating above 43°C and vice versa (10).

Despite the information about the temperature and temporal order of heat application to induce thermotolerance, there is very little known concerning the nature of the signal triggering this cellular response to heat. Also far from clear are the particular biochemical modifications within cells responsible for the cellular state of thermotolerance. Some studies have linked heat-induced cell killing and thermotolerance with perturbations of cellular GSH (11–13), a tripeptide involved in the maintenance of cellular oxidation-reduction potential (14). A possible role for oxygen radicals like superoxide in these processes has also been suggested (15).

An increase in the activity of superoxide dismutase in different types of cells was measured after exposure to hyperthermia which correlated with the development of thermotolerance. Heat also induces the synthesis of a specific set of HSPs in every organism thus far examined including cultured cells from a variety of animal sources (reviewed in Ref. 16). Development of transient thermotolerance following sublethal heat shock and its decay has been shown to correlate well with both the synthesis and the amount of HSPs (17–19). Cells treated with agents known to induce HSP synthesis also developed thermotolerance (20).

In this study, we exposed cells to the thiol compound cysteamine with or without heat in order to investigate its influence on the induction of thermotolerance for several reasons: (a) cysteamine generates activated oxygen species like superoxide and hydrogen peroxide (21, 22); (b) cysteamine leads to a rapid increase of cellular glutathione levels by a mechanism which is due to GSH biosynthesis (23); (c) thermsensitization by cysteamine of CHO cells has been reported (24, 25); and (d) this effect is dependent on the generation of activated oxygen species (26, 27).

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 was approximately 14 h during the exponential growth phase and the plating efficiency was 80 to 90%. The cells were routinely subcultured every 2 or 3 days.

Heat and Drug Exposure. Twenty-four h prior to single or split dose treatments, exponentially growing cells were trypsinized (0.25% for 2 min) and counted, and dilutions of known cell numbers (105–107 cells/flask) were inoculated in 4 replicate T25 flasks (Lux; Lab TEC) containing 4.5 ml of fresh medium (total volume). The flasks were placed in a

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2 The abbreviations used are: GSH, glutathione; CHO, Chinese hamster ovary; HSP, heat shock proteins; ΔT, min of heat treatment which reduce the surviving fraction by factor e (67%) on the exponential portion of the survival curve; TTR, thermotolerance ratio (equals the pretreated/control cell ratio of ΔT values).
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37°C incubator containing 5% CO₂ and air until treatment. Cysteamine (50 µl) was added directly to the warm medium (pH 7.4) to give a final concentration of 0.4 mM.

For initial treatments using cysteamine alone, after 30-, 60-, 90-, and 120-min drug exposure of cells at 37°C the medium was removed, and cells were washed twice with phosphate buffered saline (4.5 ml) and then covered with complete medium. Clonogenic cell survival of control cells treated under these conditions without drug was not affected (surviving fraction 95%). For initial combined drug and heat treatments, cells were exposed to cysteamine (0.4 mM) at 37°C for 15 min and then washed as indicated above. An additional 15-min heat treatment at 44°C was given thereafter (10 min) by submersing the sealed flasks in a circulating water bath (Umwaelz-Thermostat W45/EB; Haake AG, Berlin, West Germany) at 44 ± 0.05°C. For comparison, cells were initially treated with a 15-min cysteamine exposure (0.4 mM) at 44°C. As a control for the effects of heat alone, cells were exposed for 15 or 30 min at 44°C. Temperature equilibration of the medium in the flasks at the cell surface with the water bath temperature (within 0.05°C) occurred in approximately 2.5 min. The medium pH ranged from 7.4 to 7.7 throughout the experiments. If not otherwise indicated, following all individual types of initial treatment, cells were maintained at 37°C for different time intervals (7 = 2, 6, 8, and 24 h) prior to the following heat treatment at 44°C given in all cases. Control (single dose) 44°C heat treatment survival curves were obtained each time pretreated cells were exposed to following heat treatments.

Clonogenic Cell Survival. After single or split dose treatments, the cells were incubated for 8–14 days for colony development. Following incubation, the colonies were rinsed with 0.9% NaCl solution, fixed, and stained with 20% ethanol containing 0.8% ammonium oxalate and 2% crystal violet. The fraction of treated cells giving rise to colonies (>50 cells/colony) was normalized to the fraction of control cells giving rise to colonies (plating efficiency). The surviving fraction was calculated after correction for cellular multiplicity (approximately 1.9), which was determined at the time of the first initial drug and/or heat treatment. The multiplicity corrected surviving fraction data, when plotted on log (surviving fraction) versus linear (dose) paper, yielded a linear survival curve for each treated drug and/or heat exposure. The surviving fraction data arising from single or split dose treatments which reduced the surviving level to 0.15 or less were used. The slopes were expressed as D₀ values. TTR was calculated by dividing the D₀ value of the heat survival curve of pretreated cells by the D₀ (44°C) control value of cells only heated at the time of the second treatment.

If not otherwise indicated, each figure illustrates the results of one typical survival curve experiment. The error bars shown in the figures represent the standard error of 4 to 6 replicate dishes. These experiments were repeated two to five times with the same qualitative results. The D₀ values with their standard deviations and TTRs calculated from these replicate experiments are given in the tables.

Analysis of HSPs. The synthesis profiles of HSPs were analyzed as described previously (17). Briefly, at different time intervals after initial treatments (0–24 h), cells were pulse labeled with 10–20 µCi of [35S]-methionine (specific activity, >1,000 Ci/mmol; Amer sham) for 1 h at 37°C in methionine-free McCoy's Medium 5A, containing 1/100 normal levels of methionine. At the end of the labeling period, cells were rinsed three times with cold phosphate buffered saline. Sample buffer was added directly to the cells. The sample buffer contained 1% β- mercaptoethanol, 0.1% bromphenol blue, 50% glycerol, 2% sodium dodecyl sulfate, and 25% stacking gel buffer (0.5 M Tris-HCl, pH 6.8). The cell extracts were prepared by adding sample buffer to yield a final concentration of about 10⁶ cells/ml; then the extracts were boiled for 3–5 min. Equal amounts of proteins were loaded and analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel in the buffer system of Laemmli (28). Following electrophoresis, gels were fixed in 30% trichloroacetic acid, stained in Coomasie Blue G-250 in 3.5% perchloric acid, dried, and exposed directly to Kodak SB-5-X-ray film. Autoradiographs were developed by standard procedures and quantitated by scanning the film with an LKB soft laser scanning densitometer. If not otherwise indicated, each experiment was repeated twice with the same qualitative results.

Chemicals. Cysteamine hydrochloride (β-mercaptoethylamine) was obtained from Sigma Chemical Co. and stored at 5°C in a desiccator. Stock solutions were freshly prepared by dissolving the chemical in cold Dulbecco's phosphate buffered saline solution, which had previously gassed with N₂. The pH was adjusted to 7.2 to 7.4. Stock solutions of cysteamine-free methionine were sterilized by filtering through a 0.22-µm Millipore or Schleicher & Schuell membrane and kept on ice until use (1 h). Catalase from bovine liver (17,600 units/mg) was obtained from Sigma Chemical Co. Stock solution was freshly prepared in Dulbecco's solution and kept on ice.

RESULTS

Survival and Thermotolerance Response of CHO Cells Pretreated with Cysteamine and Heat at 44°C versus Heat at 44°C Alone. Initial experiments were performed to determine the influence of cysteamine upon the induction of thermotolerance by heat treatment at 44°C. The results of one typical experiment using different types of initial drug and heat treatments are given in Fig. 1. Cells were exposed to an initial treatment of 15 min cysteamine (0.4 mM) at 37°C immediately followed by a 15-min heat treatment at 44°C in the absence of drug (Treatment A). In comparison to this sequential application of drug and heat exposure, cells were simultaneously heated for 15 min at 44°C with cysteamine (0.4 mM) present during heating (Treatment B). Both types of combined drug and heat treatment reduced the surviving fraction of cells to a quite similar extent, which was 0.22 ± 0.02 and 0.09 ± 0.001, respectively. It should be noted that the surviving fraction of cells after 15 min cysteamine exposure at 37°C alone did not differ significantly from the plating efficiency of cells under these experimental conditions (see also “Materials and Methods”). A similar reduction of the surviving fraction (0.16 ± 0.01) as compared to the initial combined drug and heat treatments could be observed after a 30-min 44°C heat dose, which was used as an equivalent control for the effects of heat alone as a priming treatment (Treatment C).

Independent of the different conditioning treatments, cells became thermotolerant to a following heat treatment after a time interval (T) of 8 h at 37°C in all cases (see Fig. 1). The
second dose surviving fractions represent the results of one
typical experiment. To facilitate comparison with the control
curve, the second dose surviving fraction data have been
normalized to a surviving fraction of 1.0. When a time interval of
12 or 24 h at 37°C separated the individual treatments ($T_i = 12$
or 24 h) there was no substantial change in the expression of
thermotolerance (data not shown). For the time interval ($T_i = 8$
h), 2 to 5 independent experiments have been performed with
the same qualitative results. The control and second dose $D_0$
values as well as the TTRs indicating the degree of thermotol-
erance for these repeated experiments are listed in Table 1. At
$T_i = 8$ h, the magnitude of thermotolerance in terms of the
calculated TTR values are similarly expressed after both types
of combined treatments (TTR of 8.9 and 7.9, respectively) and
not markedly different from the effect after 30 min heat at 44°C
alone (TTR 9.3). The results further demonstrate that the
induction of thermotolerance observed after cysteamine and
44°C heat treatment is independent of the temporal order of
drug and heat application.

The influence of cysteamine combined with heat versus heat
treatment alone upon the kinetics of thermotolerance induction
in CHO cells was also investigated. The results of three inde-
pendent experiments are shown in Fig. 2. Following the initial
treatment with 15 min 0.4 mM cysteamine at 44°C, a single
second heat dose (44°C, 45 min) was given after incubation of
cells for 0 to 15 h at 37°C. By comparison with a priming
treatment of 30 min at 44°C the kinetics of induction was
similar (see Fig. 2, top). By addition of catalase (50 μg/ml)
present during the combined 0.4 mM cysteamine treatment at
44°C, thermotolerance development was comparable with the
effect of a priming treatment of 15 min heat at 44°C (see Fig.
2, bottom). The major difference in thermotolerance was
observed at a 6-h time interval by comparison between the results
shown in Fig. 2. At $T_i = 6$ h, the increase of thermal resistance
to a second heat dose was less pronounced after 15 min cyste-
amine at 44°C and 30 min heat at 44°C (Fig. 2, top) compared
to the effects of 15 min cysteamine at 44°C plus catalase and
15 min heat at 44°C alone (Fig. 2, bottom) given as priming
treatments.

Therefore, the effect of cysteamine upon the conditioning
heat treatment was further analyzed at this time interval. Fig.
3 shows the 44°C heat survival curves (0–120 min) of one
typical experiment obtained at $T_i = 6$ h after the same initial
priming treatments were given as described above. The control
and second dose $D_0$ values of 3 repeated experiments as well as
the TTRs indicating the degree of thermotolerance develop-
ment at $T_i = 6$ h are compiled in Table 2. The expression of
thermotolerance after treatment with 15 min cysteamine at
44°C or 30 min heat at 44°C was similar (TTRs of 6.6 and 5.5, Respectively). By addition of catalase (50 μg/ml) immediately
before the 15-min cysteamine treatment at 44°C, the TTR
increased (10.2) and was similar to the TTR obtained after 15
min 44°C heat treatment alone (10.8).

The possible effect of catalase alone upon the priming heat
treatments has also been checked in control (no cysteamine)
experiments. By using either heat (44°C) plus catalase (50 μg/
ml) or heat (44°C) alone as priming treatments, the $D_0$
values of the second heat survival curves were essentially identical
(data not shown).

Survival and Thermotolerance Response of CHO Cells Pre-
treated with Cysteamine at 37°C Alone. Fig. 4 shows the survival
data of CHO cells in one typical experiment after an initial
exposure to 0.4 mM cysteamine for 2 h at 37°C followed by a
heat treatment at 44°C. After a time interval of only 2 h
incubation at 37°C in the absence of cysteamine ($T_i = 2$ h), cells
became thermotolerant. The reduction of the surviving fraction
by initial pretreatment with cysteamine was approximately 10
30% under these experimental conditions. Addition of cata-
lase (50 μg/ml) prior to the addition of cysteamine and present
only for 2 h during the initial drug exposure blocked the
cytotoxic effects of cysteamine and thereby the induction of
thermotolerance. Addition of superoxide dismutase (10 μg/ml)
before cysteamine exposure could not block the induction of
thermotolerance (data not shown).

When cells were exposed to 0.4 mM cysteamine at 37°C for
different lengths of time ($t_i = min$, 37°C), the effect on
thermotolerance was dependent upon the duration of the initial
drug exposure. These results of repeated experiments are shown
in Table 3. The $D_0$ values and TTRs were obtained by regression
analysis of the exponential parts of the heat survival curves (for
further details see “Materials and Methods”).

Heat resistance acquired at $T_i = 2$ h by pretreatment with
cysteamine at 37°C enhanced the $D_0$ of the 44°C control curves

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**Table 1** Survival curve parameters ($D_0$, min) and thermotolerance ratio for 8-h time interval

<table>
<thead>
<tr>
<th>Type of pretreatment</th>
<th>No. of experiments</th>
<th>$D_0$</th>
<th>Pretreated</th>
<th>Control</th>
<th>TTR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 15 min, 37°C</td>
<td>3</td>
<td>51.1 ± 3.9</td>
<td>5.7 ± 0.7</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>0.4 mM cysteamine + 15 min, 44°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. 15 min, 37°C</td>
<td>2</td>
<td>48.4 ± 3.8</td>
<td>6.1 ± 0.5</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>0.4 mM cysteamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. 30 min, 44°C</td>
<td>5</td>
<td>56.0 ± 3.2</td>
<td>6.0 ± 0.4</td>
<td>9.3</td>
<td></td>
</tr>
</tbody>
</table>

* For definition see “Materials and Methods.”

* Mean ± SD.
by factors of 1.5, 1.6, 2.2, and 2.5 for $t_i = 30$, 60, 90, and 120 min. This increase in heat resistance observed at $T_i = 2$ h was much less pronounced after further incubation ($T_i = 8$ h) of cells in the absence of drug prior to second treatment (data not shown). The $D_o$ of the second heat survival curves of cysteamine pretreated cells ($t_i = 120$ min, $37^\circ C$) in the presence of catalase was not significantly different from the $D_o$ of the $44^\circ C$ control curves (see Table 3). Also preincubation of cells with catalase (50 $\mu$g/ml) alone at $37^\circ C$ prior to $44^\circ C$ heat treatment did not affect the clonogenic cell survival ($D_o$ of 5.2) compared to cells only heated at $44^\circ C$ ($D_o$ of 5.7).

**DISCUSSION**

The autoxidation of sulfhydryl compounds like cysteamine in the presence of oxygen involves the univalent reduction of...
Fig. 5. Autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel of [35S]methionine-labeled proteins from cysteamine (0.4 mM) and heat (44°C) treated CHO cells. Cells were exposed to cysteamine (37°C, 30 min) followed by 30 min heat at 44°C. After each type of treatment, cells were pulse labeled with [35S]methionine for 45 min, washed, and incubated with 1 nM [35S]methionine before labeling. A, actin (M, 43,000). Major HSP induction with M, 87,000, 70,000 (indicated by arrowheads) is shown.

Fig. 6. Densitometer tracing showing ability of cells to enhance the synthesis of M, 87,000 and M, 70,000 proteins following exposure to cysteamine. CHO cells were exposed to 0.4 mM cysteamine at 37°C for 30, 60, 90, or 120 min or 90 min plus catalase (50 U/ml). The profiles of protein synthesis were examined 5 h after treatment. Molecular weight (x 10^3) actin (A) are shown. HSP induction with M, 87,000 and 70,000 indicated by arrows.

Both of these activated oxygen species are freely diffusible across cellular membranes. We have previously reported that the thernosensitizing effect of cysteamine in CHO cells is based on oxidative stress induced by the sulfhydryl compound at low concentrations (25, 26). We also found a rapid increase of intracellular GSH following exposure to cysteamine at 37°C. The kinetics of thermotolerance development were similar to that observed after a priming treatment of 15 min at 44°C (Fig. 1 and Table 1). In the present study, we describe a substantial effect of cysteamine on oxidative stress induced by the sulfhydryl compound at low concentrations known to affect intracellular GSH (25, 26). We also found a rapid increase of intracellular GSH following exposure to cysteamine at 37°C. The kinetics of thermotolerance development were similar to that observed after a priming treatment of 15 min at 44°C (Fig. 1 and Table 1).
exposure was markedly changed (see Fig. 2). The effect of catalase is most probably explained by scavenging H₂O₂ and thereby blocking the sensitizing effect of cysteamine upon the following heat exposure in these experiments. In a comprehensive study, Majima and Gerweck (4) showed a substantial sparing effect due to dose fractionation of the initial heat treatment. By comparison with their results, we found that the time needed to reach expression of thermotolerance increased similarly with decreasing survival levels after priming treatments. Inhibition of the cysteamine effect by catalase was substantial at T = 6 h, where also the sparing effect due to either heat treatment alone or combined drug and heat treatment was most pronounced.

The observed dose-dependent increase in the heat resistance of CHO cells pretreated with cysteamine at 37°C is especially interesting with regard to the mechanisms of thermotolerance induction. It has been reported previously that Salmonella typhimurium pretreated with H₂O₂ become resistant to killing by heat (31). This adaptation was accompanied by the enhanced synthesis of a set of proteins, three of which were also induced by heat shock. More recently, a slight but significant increase in resistance to 43°C heat treatment was found to be induced in CHO cells following H₂O₂ pretreatment (32). The data strongly suggest that activated oxygen species like H₂O₂ play an important role in the molecular process triggering thermotolerance. Our results support this idea, since the addition of catalase blocked the induction of heat resistance by cysteamine at 37°C. However, with regard to the mechanism, the rapid elevation of intracellular GSH by thiols (23) which parallels the thermosensitizing and cytotoxic effects of such compounds (25, 26) might be equally important. GSH elevation during the induction of thermotolerance has been reported by Mitchell et al. (12, 13). Also, reduction of its development and decrease in HSP synthesis could be observed by GSH depletion and prevention of GSH biosynthesis prior to heating (11, 12). In the case of cysteamine, it seems possible that the interaction of elevated GSH content with generated oxygen species like H₂O₂ after cysteamine treatment causes significant perturbation of the intracellular GSH system and thereby triggering of the induction of thermotolerance. The formation of glutathione disulfide following cysteamine treatment could be an important signal for further modulation of protein synthesis similar to the substantial effects of glutathione disulfide on the initiation of protein synthesis in rabbit reticulocytes (33). The kinetics of cellular GSH increase and its decay after cysteamine treatment (23) is similar to the observed induction of heat resistance in the study which occurred within 2 h after cysteamine treatment. These results further support the idea that the induced increase in GSH may contribute a major effect in the signal for the induction of heat resistance by cysteamine. The interaction of GSH and H₂O₂ in this mechanism might also explain why the kinetics of heat resistance after H₂O₂ treatment alone differed significantly from our results with cysteamine (32). Our observation that cysteamine exposure at 37°C also leads to slight increase in the synthesis of HSP (e.g., M, 70,000 and M, 87,000) allows us to study in more detail the modulation of HSP synthesis by oxidative stress under these conditions. It seems most likely that the reported induction of HSP in various mammalian cells by sulhydryl reagents, transition series metals, chelating agents (34, 35), and superoxide (36) might be based on a common mechanism via oxidative stress. Such agents like cysteamine can act as components of a Fenton type reaction (37), a chemical process which generates highly reactive oxygen species in a metal catalyzed reaction.

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