Interaction of Hyperthermia and Metabolic Inhibitors on the Induction of Chromosome Damage in Chinese Hamster Ovary Cells

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ABSTRACT

We have examined the chromosomal effects of heat causing asynchronously growing Chinese hamster ovary (CHO K1) cells in the presence of actinomycin D or cycloheximide. Actinomycin D was found to strongly potentiate the chromosome damaging effects of heat shock, an effect correlated with a strong nonadditive reduction in cell survival. In contrast, cycloheximide treatment reduced heat shock induced chromosome damage and resulted in a significant nonadditive increase in cell survival following heat shock. The different effects of these two inhibitors on chromosomal damage and cell survival are correlated in part with their effects on the rate of DNA synthesis during heat shock. The results suggest that an important aspect of the interaction of heat and metabolic inhibitors involves changes in cell cycle phase distribution of and/or progression through the S phase of the cell cycle induced by drug treatment prior to and during heat shock. The data indicate that the protective effect of cycloheximide in heat shocked cells may involve altered cell cycle progression and/or phase distribution of cells during hyperthermia.

INTRODUCTION

Recent experiments in which cells were treated with cycloheximide before and during heat shock have shown that cell survival can be enhanced under conditions in which protein synthesis is blocked (1) and that thermostolerance can be induced in the absence of HSP expression (2-5). These data have important implications for the hypothesis that thermostolerance requires the expression of HSPs (6), as well as the general assumption of a protective role for the heat shock proteins. However, the mechanism by which cycloheximide acts to protect cells from heat induced damage and how it influences the thermostolerant state remains unknown.

Heat shock induces chromosome damage in mammalian cells (7, 8), and heat treatment of cells in S phase results in higher levels of chromosome damage than in other cell cycle phases, an effect correlated with the S-phase sensitivity of cells to the killing effects of heat (7-10). It has been proposed that the S-phase sensitivity of mammalian cells to heat is directly due to DNA damage occurring during hyperthermia (10, 11).

We have examined the effects of treating cells with cycloheximide during heat shock and find that chromosomal damage is reduced and cell survival enhanced when cycloheximide is present during a single treatment with a 43°C heat shock in asynchronously growing CHO K1 cells. In contrast, low dose actinomycin D treatment strongly potentiated both the chromosome damaging effects of heat and cell killing. The contrasting effects of these drugs appear to be correlated with their effects on the rate of DNA synthesis and indicate that at least with respect to heat induced genomic damage and the S-phase sensitivity of mammalian cells to hyperthermia, drug induced alterations in cell cycle progression may be an important factor in altering lethal effects of heat shock and in explaining the protective effect of cycloheximide on heat shocked cells.

MATERIALS AND METHODS

Cell Culture. CHO K1 cells were grown in Ham's F-12 medium supplemented with 10% dialyzed fetal bovine serum, penicillin, and streptomycin but lacking thymine, glycine, and hypoxanthine. The cells were confirmed to be free of Mycoplasma contamination by direct microscopic examination of Hoechst-stained cells.

Heat Shock Treatments. Asynchronously growing cells were heat shocked for variable lengths of time at 43.0°C in a tissue culture incubator. Heat shock was administered by placing 100-mm plates (7 ml of medium) into dishes of water maintained at 43°C in a 5% CO2 high humidity atmosphere. The medium attained heat shock temperature within 10 to 15 min of placement in the incubator, and the standard treatment was for 75 min. Following heat shock, the cells were returned to 37°C to equilibrate for 20 min prior to changing medium.

Drug Treatments. For combined drug and heat shock treatment, drugs were added as a concentrated stock 30 min prior to heat shock, and the cells were maintained at 37°C. Following heat shock, drugs were maintained in the medium for 20 min before the drug was removed by washing cells twice with prewarmed fresh medium.

Determination of Cell Survival. Cell survival was measured by colony formation after heat shock. From 100 to 200 cells were plated and allowed to attach for 1 to 2 h before heating. Cells treated with drugs only were exposed at this time for equivalent lengths of time. Following heat shock, cells were washed twice with prewarmed medium, 10 ml of fresh medium were added, and colonies were allowed to grow until large enough to count (>50 cells/colony). Colonies were then fixed, stained with Giemsa, and counted. Cells treated with drugs were handled as described above.

Measurement of the Rate of DNA Synthesis. The rate of DNA synthesis was monitored by measuring the incorporation of [H]dThd into trichloroacetic acid precipitable material. During and following treatment of cells with heat and/or drug, cells were incubated for 20 min with medium containing [H]dThd (2 μCi/ml; 6.7 Ci/mmol; American). Three replicates per time point were labeled for each treatment. Labeling was terminated by addition of ice-cold phosphate buffered saline, and the cells were immediately lysed in 1 ml of buffer (1 mM EDTA—0.5% sodium dodecyl sulfate—10 mM Tris, pH 8.0). Trichloroacetic acid was added to 5%, and the precipitated material was collected on Whatman GF/C filters and counted.

Preparation and Analysis of Metaphase Chromosomes. Metaphase chromosome spreads were prepared 18—24 h following treatment of cells. Colcemid was added to the incubation medium and cells were harvested 1 h later. Air-dried slides were stained with 4% Giemsa and metaphase spreads were examined and photographed on an Olympus microscope.

Numerous types of chromosome damage were observed in heat shocked cells. Chromatid breakage was relatively easy to quantify and was, therefore, used to measure the extent of damage per cell. Aberrations scored as chromatid breaks included unequivocal breaks, chromatid exchanges, and large chromatid deletions with displacement of chromatid ends (see Fig. 1). An arbitrary scale of damage per cell was utilized. Briefly, cells were scored as: 1—4 breaks, damage of level 1 severity; 5—10 breaks per cell, level 2; more than 10 breaks, level 3 damage. Cells which displayed karyotypic abnormalities such as chro-
Fig. 1. Chromosome damage induced by heat shock in the presence of actinomycin D (0.1 \mu g/ml). Cells were heat shocked with inhibitor, and metaphase cells were harvested after 18–24 h of recovery in drug free medium. A, control cell with no gross chromosomal abnormalities; B, cell after combined treatment showing 7 chromatid breaks including one chromatid exchange (arrows).

matid or chromosome gaps, fragile site expression, or uncondensed chromosomal segments, but not actual chromatid breakage, were classified as having “minor” damage. Extreme damage, e.g., chromosomal fragmentation, was scored as level 3 damage.

RESULTS

Effects of Metabolic Inhibitors on the Survival of Heat Shocked Cells. The survival of CHO K1 cells heat shocked for different times at 43°C declines with increasing duration of exposure to heat as shown in Fig. 2. Each point in this figure represents the mean of at least four experiments. Cell survival was strongly affected by the presence of metabolic inhibitors. Cycloheximide alone at a concentration of 10 \mu g/ml had only a very small effect on cell survival, reducing it to approximately 90% of control. This effect did not change significantly over the range of treatment durations examined (Fig. 2). Prolonged exposure to cycloheximide alone (18 h) reduced survival to 44% of control (data not shown). Actinomycin D itself is highly toxic to cells, and the survival of cells exposed to 0.1 or 0.2 \mu g/ml actinomycin for even relatively short periods of time was reduced significantly (Fig. 3).

As a null hypothesis, the combined effects of heat shock and drug treatment were predicted to be simply additive. Predicted additive curves for our conditions are shown in Figs. 2 and 3 and were calculated taking into account the fact that cells were

Fig. 2. Effect of cycloheximide treatment on survival of heat shocked cells. Cells were heat shocked in the presence of 10 \mu g/ml cycloheximide as described in “Materials and Methods” and colonies were counted after 7–10 days of growth. ■, heat shock alone; ▲, cycloheximide alone; △, heat shock plus cycloheximide; ○, expected additive survival.

Fig. 3. Effect of actinomycin D on survival of heat shocked cells. Cells were heat shocked in the presence of 0.1 \mu g/ml actinomycin D and colonies were counted after 7–10 days of growth. ■, heat shock alone; ▲, actinomycin D (0.1 \mu g/ml) alone; ○, actinomycin D (0.2 \mu g/ml) alone; △, heat shock plus actinomycin D (0.1 \mu g/ml); ○, expected additive survival for heat shock plus actinomycin (0.1 \mu g/ml).
exposed to drugs 50 min longer than to heat shock. When cells were heat shocked in the presence of cycloheximide, cell survival was reduced to about 85% of control and this value did not decrease significantly with increasing duration of heat shock (up to 2 h of treatment). This represents significantly greater survival than expected for additive treatment effects. This effect is also seen after puromycin (1) treatment indicating that general inhibition of protein synthesis, or some effect of that inhibition, enhances cell survival after heat shock.

In contrast to the combined effect of heat and cycloheximide, cell killing was strongly potentiated by low concentrations of actinomycin D. When cells were heat shocked in the presence of 0.1 µg/ml actinomycin D, cell survival was reduced far below that expected for predicted additive effects of heat shock and actinomycin (Fig. 3). Cell survival was reduced to less than 0.5% of control when cells were heat shocked in the presence of 0.2 µg/ml actinomycin D for more than 1 h.

Metabolic Inhibitors Alter the Chromosome Damaging Effects of Heat Shock. Metaphase cells were not abundant in cell populations heat shocked for 75 min at 43°C until 18–24 h after heat shock. This was also true for cells heat shocked in the presence of cycloheximide or actinomycin D. Presumably, this reflects the transient block in cell cycle progression induced by heat shock (12, 13) and so as a consequence we routinely prepared metaphase spreads at this time.

In heat shocked cells, the frequency of metaphases with damaged chromosomes was 20–30% of total metaphases observed (Table 1). While cells with aberrant metaphase chromosomes were relatively frequent, the degree of damage was generally low, with about one-half of the damaged cells showing only minor damage and only 8–10% being severely damaged (level 2 and 3 damage (Table 1)). An example of severe chromosomal damage is shown in Fig. 1.

Cycloheximide (10 µg/ml) significantly reduced the frequency and extent of chromosomal damage in heat shocked cells. Following combined treatment, the frequency of damaged metaphases was less than 10%. In addition, the extent of damage per cell was significantly reduced so that typically cells displayed only minor damage and only 8–10% being severely damaged (level 2 and 3 damage (Table 1)). Thus, the increased survival of cells heat shocked in the presence of cycloheximide is correlated with a reduction in heat induced DNA damage.

The combined effects of heat shock and actinomycin D (0.1 µg/ml) is shown in Table 1. As with cell killing, combined treatment resulted in greater than additive damage. The frequency of chromosomally damaged cells was increased over 2-fold by the addition of actinomycin, and, more significantly, the extent of damage per cell was increased 7-fold (8.6% of the cells with level 2 and 3 damage in heat shocked cells versus 60.2% in cells heat shocked in the presence of actinomycin (see Table 1). At 0.2 µg/ml, metaphase cells were extremely rare after the 43°C heat shock, and the few which were observed exhibited severe damage. Actinomycin alone induced a significant level of chromosomal damage in CHO K1 cells (Table 1).

Interaction of Metabolic Inhibitors and Heat Shock on DNA Synthesis. We examined the possibility that the contrasting effects of cycloheximide and actinomycin on heat shock induced chromosome damage (and perhaps secondarily on cell survival) might reflect a differential effect of these inhibitors on cell progression into and/or through S phase, i.e., on the rate of DNA synthesis at the time of heat shock.

Cycloheximide inhibits DNA synthesis in mammalian cells (14–16) and we observed that [3H]thymidine incorporation was inhibited 90–95% in unheated asynchronously growing CHO K1 cells (Fig. 4). This inhibition occurred rapidly after addition of the drug and was also reversed rapidly following removal of the drug. Actinomycin D, at a concentration of 0.1 µg/ml, had significantly less effect on the rate of DNA synthesis, reducing it only 20–30% following a brief period of enhanced thymidine incorporation (110–130% of control (Fig. 4)). Thymidine incorporation was never observed to fall below 60–70% within 8 h of a 75-min pulse of actinomycin (0.1 µg/ml). Heat shock inhibits DNA synthesis in mammalian cells (11, 17) and we observed heat shock to reduce thymidine incorporation 80–90% with maximal inhibition of DNA synthesis occurring 4–8 h after heat shock (Fig. 5). Recovery did not begin until at least 8–10 h after heat shock (data not shown).

The effect of cycloheximide and actinomycin on the inhibition of DNA synthesis observed in heat shocked cells is shown in Fig. 5, which summarizes the results of three experiments. Cycloheximide added 30 min prior to heat shock reduced the rate of DNA synthesis to 10% of control so that the cells progressed through S phase at a very reduced rate at the onset of heat shock. Heat shock caused a slight increase in thymidine incorporation in cycloheximide treated cells, but the rate of

![Fig. 4. Rate of DNA synthesis in cells treated with cycloheximide or actinomycin D. Cells were pulse-labeled for 20 min with [3H]thymidine during and after treatment with drug, and acid precipitable material was counted. Rate of thymidine incorporation expressed relative to untreated controls.](cancerres.aacrjournals.org)
DNA synthesis remained significantly below cells heat shocked without inhibitor as long as the drug was present in the medium. Cycloheximide also had the effect of accelerating the rate at which DNA synthesis recovered after heat shock so that thymidine incorporation in cells heat shocked in the presence of drug exceeded that in heat shock controls within 1–1.5 h after heat shock and removal of the drug (Fig. 5). Actinomycin D, however, transiently stimulated DNA synthesis such that, under the experimental protocol utilized (30 min drug treatment before heat shock), DNA synthesis was proceeding at a rate about 115% of control (approximately 15-fold higher rate than in cycloheximide treated cells) at the time heat shock began (Fig. 5). The rate of heat shock induced inhibition of DNA synthesis was slowed relative to heat shock controls, so that \([^3H]dTdT\) incorporation was 25–50% higher in actinomycin D treated cells than in heat shock controls. Thus, cycloheximide and actinomycin have contrasting effects on DNA synthesis and significantly alter the normal pattern of cell progression into and/or through S phase during and after heat shock.

We wanted to determine whether the effects of cycloheximide on heat induced chromosome damage could be mimicked by a specific inhibitor of DNA synthesis and so we treated cells with aphidicolin (1 \(\mu\)g/ml) during heat shock as described. We found that chromosome damage was reduced nearly as much as with cycloheximide (Table 1). At this concentration of aphidicolin, this effect is time dependent because prolonged treatment with the drug results in a large potentiation of cell killing and chromosome damage correlated with “escape” from the DNA synthesis inhibition and the synchronous movement of cells into early S phase.\(^5\)

**DISCUSSION**

As observed by others (1–4), we have found that treating asynchronously growing cells with cycloheximide protects them from lethal damage induced by a single dose heat treatment. Our data show that this increase in survival is correlated with a significant reduction in the frequency of cells with chromosomal damage as well as a reduction in the extent of damage per cell. Enhancement of cell survival after moderate heat shock in the presence of protein synthesis inhibitors has been shown to be as large as 4 logs in magnitude with prolonged heat shock (1). While our data show that the reduction in the frequency of cells with chromosomal damage and the increase in cell survival are of similar magnitude (30%), cell survival after heat shock (with cycloheximide) was about 85% while the frequency of cells with chromosomal damage was only 2%, indicating that heat induced cell killing involves factors other than chromosomal damage.

Several cytotoxic drugs, including actinomycin, have been shown to interact with hyperthermia to potentiate cell killing (18, 19). We have found that with actinomycin treated cells, this effect is correlated with a large increase in the frequency and extent of chromosomal damage after heat shock. While it is clear that chromosome damage is greater in cells treated with heat and actinomycin than in cells treated with either actinomycin alone or heat alone, the toxicity of actinomycin (including clastogenic effects) precludes ascribing specific mechanisms to the observed increased genomic damage. Other studies have concluded that enhanced cell killing with combined heat-actinomycin treatment involves altered membrane permeability induced by heat (19).

Our original intent was to use cycloheximide and actinomycin to examine the chromosomal effects of heat shock when HSP expression during heat shock was inhibited. In experiments not reported here, we found that both drugs completely (cycloheximide) or partially (actinomycin) inhibited HSP expression immediately after heat shock. However, the two drugs led to contrasting effects on chromosome damage suggesting that HSP expression per se was relatively unimportant with respect to heat induced genome damage under the conditions we used. Therefore, we measured the rate of DNA synthesis (\([^3H]dTdT\) incorporation) during combined heat and drug treatment to determine whether alterations in the frequency and extent of chromosome damage were correlated with different rates of cell entry into S phase and/or progression through S phase in cells heat shocked in the presence of drugs. Cycloheximide blocks cell cycle progression in all phases (reviewed in Ref. 20) and rapidly inhibits DNA synthesis (14–16). In our experiments DNA synthesis was strongly inhibited prior to heat shock (Fig. 5) and thus cells were initiating DNA synthesis, and proceeding through S at a very reduced rate at the time of heat shock. This was true also for cells treated with aphidicolin. Inhibition of DNA synthesis with hydroxyurea has been shown to enhance cell survival at “low” heat doses, but not following severe (45°C) hyperthermia (17). The protective effect of cycloheximide is also known to be reduced with severe heat treatment (1). While we have not examined the chromosomal effects of more severe heat shock, our data would seem to indicate that the S-phase sensitivity of DNA to damaging effects of heat, at least under conditions used here, does not necessarily reflect the direct thermal damage to DNA, but rather damage to activity replicating DNA, as has been suggested (11).

Actinomycin D has been reported to block the G1–S transition but not inhibit DNA synthesis in cells already in S phase (21, 22). We observed a transient stimulation of thymidine incorporation after addition of actinomycin and a subsequent reduction in DNA synthesis of only 10–20%. We do not know if G1–S progression was affected by actinomycin, but clearly DNA

synthesis continued at a much higher rate prior to heat shock than in cycloheximide treated cells. The data also indicate that DNA synthesis during heat shock continued at a somewhat higher rate in actinomycin treated cells than in either cycloheximide treated cells or heat shock controls. It is not clear, however, whether this rate differential during heat shock is biologically significant. The potentiation of heat induced cell killing by actinomycin, however, clearly involves factors other than alteration of the proportion of cells in S phase during heat shock because the percentage of cells killed is greater than the fraction of cells which would enter and traverse or enter S during a 75-min heat shock. These data may be of some significance insofar as they indicate that drug induced changes in cell cycle phase distribution and/or progression may be a significant factor influencing cell killing effects of hyperthermia at temperatures similar to those used clinically.

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