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CYCLOHEXIMIDE INCREASES THE THERMOSTABILITY OF PROTEINS IN CHINESE HAMSTER OVARY CELLS

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Protein denaturation resulting from temperatures between 42.0°C and 50°C has been observed and implicated as the lethal lesion for hyperthermic cell killing. A logical corollary is that protection against hyperthemic killing requires stabilization of cellular proteins against thermal denaturation. To test this, Chinese hamster ovary cells were treated with the heat protector cycloheximide and then subjected to differential scanning calorimetry to measure protein denaturation. Cycloheximide stabilized proteins that denatured between 42°C and 52°C in control cells by increasing their transition (denaturation) temperature by an average of 1.3°C. In addition, cycloheximide reduced the cytotoxicity of actinomycin D and adriamycin, suggesting that protein stabilization protects cells against stresses other than hyperthermia.

Time-temperature analyses of hyperthermic cell killing yield an activation energy for mammalian cell killing of 502 KJ/mole to 586 KJ/mole (1,2), as calculated using the Eyring hypothesis (3). Since this is similar to the activation energy obtained for the thermal denaturation of a wide range of proteins (1,3), it has been suggested that protein denaturation is a lethal result of hyperthermia (1,2). Differential scanning calorimetry (DSC) has demonstrated that hyperthermia does denature mammalian cell proteins with phase transition temperatures (Tm) between 40.0°C and 55.0°C (4,5). The critical target that represents the lethal hyperthermic lesion, i.e., the rate limiting step, would appear to be one or more proteins that denature between 40.0°C and 55.0°C. When critical target analysis was applied to the data, a prediction that the transition temperature of the critical target proteins is near 46.0°C was obtained (4,5).

Treatments that sensitize cells to hyperthermia, e.g., short chain alcohols (6,7), decreased Tmc (4) while treatments that protect

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cells, e.g., glycerol (8) and thermotolerance, increased Tmc by 1.0°C to 1.3°C (5). These data supported the hypothesis that protein denaturation is a lethal hyperthermic lesion.

Treating cells with 10 ug/ml cycloheximide (CHM) 1 h before and then during hyperthermia provides the same degree of protection against cell killing at 43.0°C as glycerol and thermotolerance, and removing CHM prior to heating reduces the protection markedly (9). CHM enters cells within minutes (10) but requires 1 h to induce thermal protection. This implies that CHM itself does not protect cells but initiates some intracellular changes that result in protection.

If protein denaturation is the lethal lesion for hyperthermic cell killing CHM treatment should cause a shift in the DSC scan of whole cells similar to that induced by glycerol and thermotolerance. Conversely, rinsing out CHM prior to scanning should reduce this shift. To test this hypothesis, Chinese hamster ovary (CHO) cells were treated with CHM and then subjected to DSC. The results support this hypothesis.

It has been reported that thermotolerance provides protection against several chemotherapeutic drugs (11,12). If the protection was due to protein stabilization then CHM should also provide protection. When applied 1 h before and then during treatment with either actinomycin D (AD) or adriamycin (ADM), CHM did provide substantial protection against cell killing. This finding implies that protein stabilization provides protection against stresses other than hyperthermia and may represent a component of some more general cellular stress response.

MATERIALS AND METHODS

Cell Culture and Survival Determination

Chinese hamster ovary (CHO) cells were grown as either suspension (13) or monolayer (9) cultures in McCoy's 5A medium. The medium contained 10% iron-supplemented calf serum, 26 mM sodium bicarbonate, 0.1 g/l streptomycin sulphate, and 0.07 g/l penicillin G potassium. Spinner flasks were gassed with CO_2 in order to adjust the pH to 7.4 (13) before being placed in a 37.0°C warm room. Monolayer cultures were maintained in a 37.0°C humidified incubator containing a mixture of 95% air and 5% CO_2. For survival determination, cells were diluted appropriately (following trypsinization for monolayer cells) and plated into 25 cm^2 culture flasks containing lethally irradiated feeder cells (4,000 feeder cells/cm^2) (14). After 1-2 weeks of incubation, colonies were stained and counted.

Drug Treatments

Concentrated stock solutions of actinomycin D (AD) or adriamycin (ADM) were made in DMSO and diluted 1,000-fold in medium. The final DMSO concentration of 0.1% has been shown to be noncytotoxic (15). Experiments with AD and ADM were performed with monolayer cultures.

After incubating cells with the drug for the prescribed time at 37.0°C, the drug was removed with three rinses of medium, after which the cells were trypsinized and subjected to the colony formation assay.

CHM (10 ug/ml) was administered for 1 h at 37.0°C prior to and then during one of the following procedures: DSC, 43.0°C hyperthrmia, or chemotherapeutic drug treatment at 37.0°C. When cells were to be treated with CHM and another drug, the second drug was administered to the cells in medium containing CHM.

Glycerol Treatment and Thermotolerance Induction

Cells were incubated for 0.5 h, at 37.0°C, in medium containing 5% glycerol, and subsequently heated in this medium.

Thermotolerance was induced by heating the cells for 12 min at 43.0°C and then incubating them at 37.0°C for 3 h before the second heat treatment.

Hyperthermia Treatment

Cells were heated in suspension at a concentration of $3x10^5$ cells/ml. Five ml of the suspension was added to a 15 ml centrifuge tube. After gassing the tube with a mixture of 5% CO_2 and 95% air, its cap was tightened and sealed with paraffin film. The tube was placed (horizontally) into a rack that was submerged in a 43.0°C water bath. A wrist action shaker was used to agitate the cells during hyperthermia.

Differential Scanning Calorimetry

Differential scanning calorimetry was performed on live CHO cells according to the procedure described by Lepock et al. (4,5). Between 3.5×10^8 and 3.8×10^8 cells were used per scan, the scan rate was 1.0°C/min, and each scan was repeated four times. The intrinsic instrumental baseline curvature and increase in Cp of denaturation (Cp) were corrected, as described previously (15).

RESULTS AND DISCUSSION

Treating Chinese hamster ovary (CHO) cells with 10 ug/ml cycloheximide (CHM) 1 h before and then during hyperthermia at 43.0°C provided the same degree of protection against cell killing as glycerol and thermotolerance (Fig. 1). When CHM was removed 30 min prior to heating, protection was reduced markedly (9) (Fig. 1).

Averaged DSC scans for experimental and control cells are presented in Fig. 2. The averaged DSC scan of CHM-treated cells exhibited a shift towards higher temperatures with respect to that of control cells. The shift was most pronounced in the region between 42°C and 55°C and indiscernible at temperatures above 65.0°C (data not shown). The transitions above 65°C occurred at similar Tm values as those observed in V79 cells with no significant shifts induced by CHM, as was true in the case of thermotolerant cells (4,5). At the 46.0°C point on the control curve (Tmc) the shift was 0.8°C ± 0.4°C, and the average Tm of peak A (48°C to 52°C on control curve) was increased by 2.0°C. When CHM was removed and the cells allowed to incubate in fresh medium for 30 min prior to DSC (no CHM during DSC) the CHM-induced

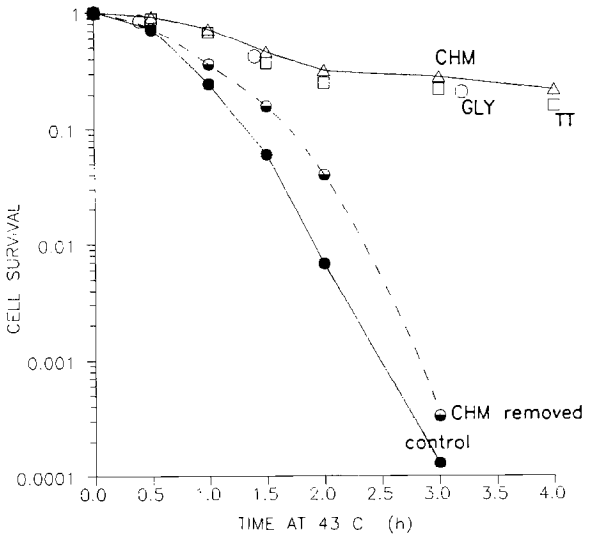

Figure 1. Survival curves for CHO cells heated at 43.0°. The protective effects of cycloheximide (CHM), glycerol (GLY), and thermotolerance (TT) are illustrated. When CHM was removed 30 min before heating, most of its protective effect was eliminated.

shift of the DSC scan was: eliminated between 42°C and 48°C, halved between 48°C and 55°C, and unaffected between 55.0°C and 65°C (Fig. 2).

DSC scans of isolated cellular organelles have shown that order-disorder transitions of cytoplasmic proteins contribute largely to peak A and those of membrane proteins to peak B. However, all cellular components and organelles contain some thermolabile proteins

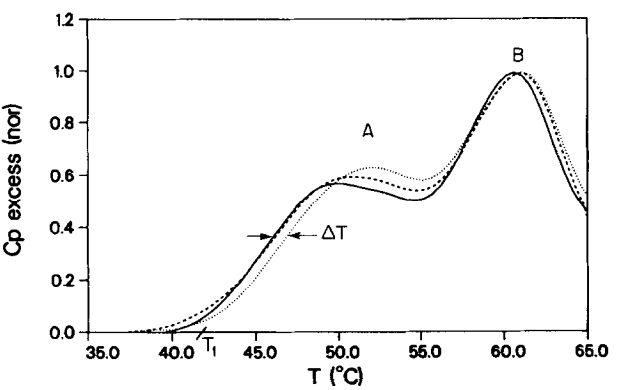

Figure 2. Differential scanning calorimetry scans (excess specific heat (Cp) versus temperature) of whole CHO cells. This figure shows the scans from 35.0°C to 65.0°C for: 1) control cells (————————), 2) CHM-treated cells (·····), and 3) cells for which CHM was removed 30 min before the scan (----------). Each curve is the average of four independent scans. The intrinsic instrumental baseline curvature and increase in Cp of denaturation were corrected, as described previously (16).

denaturing under the A peak ($40^{o}C$-$55^{o}C$) (Lepock et al., unpublished data) and are thus potential candidates for the critical target.

The shift in the DSC profile at Tmc ($0.8^{o}C \pm 0.4^{o}C$) is not significantly different from the predicted shift in Tmc of the critical target ($1.0^{o}C$) and similar to that observed previously for thermotolerant and glycerol-treated V79 cells (4,5). For CHM-treated cells there are two possible mechanisms for the shift: 1) a depletion of the most thermolabile proteins (possibly newly synthesized proteins) during the 1 h incubation with CHM (17), or 2) protein stabilization detectable as an increase in the Tm of the most thermolabile proteins. The shift in the Tm of peak A supports the latter of these two mechanisms.

The similarity in the degree of thermal protection provided by glycerol, thermotolerance, or CHM, at $43.0^{o}C$, suggests a common mode of thermal protection. The observation that all three treatments increased the thermostability of proteins with Tm values between $42^{o}C$ and $65^{o}C$ suggests that this is the protective mechanism.

Glycerol is known to stabilize proteins in a direct manner (5,18). This does not appear to be the case with CHM. CHM enters cells rapidly, establishes an intracellular steady state concentration within 2 min (10), and inhibits protein synthesis to 5% of control levels within 10 min (9). Yet, at least 1 h was required for maximum thermal resistance to develop in CHO cells. In this respect, CHM is more like thermotolerance, i.e, thermal protection is not manifested immediately. CHM may initiate some action by the cells that leads to protein stabilization, and subsequently, thermal protection.

The trigger for the cellular action may be the stress of protein synthesis inhibition. Support for this hypothesis is provided by the fact that other protein synthesis inhibitors, viz., puromycin (9) and histidinol (19) also induce thermoprotection. There are no chemical structural similarities between CHM, puromycin, and histidinol, and all three inhibit protein synthesis by different mechanisms. Hence it is unlikely that these substances induce thermal resistance by a direct chemical interaction; more likely, resistance is a secondary response resulting from their common effect of protein synthesis inhibition. This secondary response may be a stabilization of thermolabile cellular proteins to prevent their destruction by normal turnover processes or by thermal denaturation.

Cycloheximide also protects against the cytotoxicity of two chemotherapeutic drugs, actinomycin D (AD) and adriamycin (ADM) (Figs. 3a and 3b). Hence, the CHM-induced cellular changes that lead to

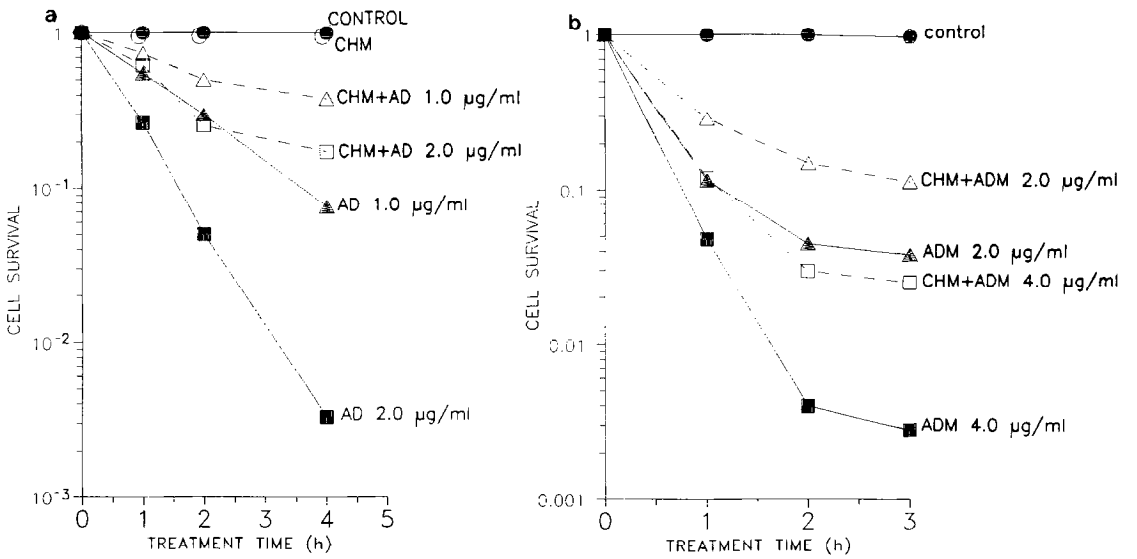

Figure 3. Protective effects of cycloheximide (CHM) against: a) actinomycin D and b) adriamycin cytotoxicity. The drug concentrations used are indicated in the figures. Figure 3a also indicates that CHM treatment alone had no effect on cell survival.

thermoprotection appear to convey resistance to other stresses. Thermotolerance protects against AD and ADM cytotoxicity (11,12) to a similar degree as CHM treatment. The observations that CHM and thermotolerance induce the same magnitude of protection against heating at 43.0°C and against two chemotherapeutic drugs further suggest that protection is induced by a similar mechanism. Clearly, heat shock protein synthesis cannot be the common factor since CHM inhibits protein synthesis, including that of heat shock proteins (20,21). Protein stabilization, as evidenced by DSC, is a common result of both CHM treatment and thermotolerance and is a prime candidate for the mechanism that induces both thermoprotection and resistance to these cytotoxic drugs. The latter may result from the targets of the drugs being stabilized against chemical interaction with the drugs. In support of this contention, initial experiments have demonstrated that CHM reduced, by four-fold, the amount of [^{3}H]-actinomycin D that associated with the TCA-precipitable cell fraction (M.J. Borrelli, unpublished data). This fraction contained the DNA, the site in the cell where AD binds (22).

Protein stabilization is associated with treatments that impart protection to a variety of stresses and may represent one aspect of a general cellular response to stress that may involve: 1) heat shock proteins already present within cells, 2) post-translational modification of cellular proteins, 3) the synthesis of non-

proteinacious protective substances, and/or 4) reassociation of cellular proteins resulting in stabilization due to increased protein-protein interactions.

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