Hyperthermia and protein aggregation
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THERMOTOLERANCE AND NUCLEAR PROTEIN AGGREGATION: PROTECTION AGAINST INITIAL DAMAGE OR BETTER RECOVERY?

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ABSTRACT

Heat-induced nuclear protein aggregation and subsequent disaggregation were measured in non-preheated and preheated (thermotolerant) HeLa S3 cells. The effect of thermotolerance on the formation of and recovery from heat-induced nuclear protein aggregates was related to the cellular levels of hsp27, hsp60, hsp70, hsc70 and hsp90. Cells heated at different time points after the thermotolerance trigger showed various levels of protection against heat-induced nuclear protein aggregation. This protection however, did not parallel the development and decay of thermotolerance on cell survival. The protection was maximal when the thermotolerance level already had started to decay. The level of protection against nuclear protein aggregation did however parallel the cellular level of hsp70 indicating that hsp70 may be involved in this process. At all stages during the development and decay, thermotolerant cells showed a more rapid recovery (disaggregation) from the heat-induced nuclear protein aggregates than non-thermotolerant cells. The rates of disaggregation during development and decay of thermotolerance paralleled the cellular levels of hsp27 suggesting that hsp27 is somehow involved in this recovery process from heat-induced nuclear protein aggregates. The total cellular levels of none of the individual hsp’s completely correlate with development and decay of thermotolerance, indicating that overexpression of any of these hsp’s alone does not determine the level of thermotolerance. Clonogenic cell survival paralleled the rates of disaggregation, leading to the notion that recovery processes are the most important determinant for the thermotolerant state of HeLa S3 cells. The best correlation with clonogenic survival was found when both initial aggregation and subsequent disaggregation were taken into account, suggesting that the combined action of various hsp’s in these two processes have to be included in thermotolerance development and decay.
6.1 INTRODUCTION

Exposure of cells to hyperthermia can induce a transient resistance (thermotolerance (TT)) to a subsequent heat treatment [Gerner and Schneider 1975]. There is strong evidence that heat shock proteins are involved in the acquisition of thermotolerance [Landry et al. 1982, Li and Werb 1982, Subjeck et al. 1982, Lindquist and Craig 1988]. Good correlations were observed between the cellular levels of hsp’s and the development and decay of thermotolerance, especially for hsp27 [Landry et al. 1991] and hsp70 [Li and Werb 1982, Li 1985, 1989]. Recently, also hsp40 has been shown to correlate well with the acquisition of thermotolerance. The involvement of hsp’s in thermoresistance is supported by the observations that (stable) transfection of cells with individual genes coding for hsp27 [Landry et al. 1989] and hsp70 [Li et al. 1991, Angelidis et al. 1991] lead to a lower vulnerability to the killing effect of heat. Competitive inhibition of heat-induced hsp expression by introduction of a large number of copies of the heat shock element results in increased heat sensitivity [Johnston and Kucey 1988]. Also, microinjection of cells with antibodies against hsp70 had a thermosensitizing effect [Riabowol et al. 1988].

The most likely trigger for hsp synthesis and thermotolerance induction are denatured/aggregated proteins [Finley et al. 1984, Hahn and Li 1990, Hightower 1991, Kampinga 1993]. Microinjection with denatured proteins resulted in hsp synthesis and thermotolerance development [Ananthan et al. 1986]. Also puromycin, causing premature chain termination during protein synthesis, and intracellular protein crosslinking by diamide induced the development of thermotolerance [Lee and Dewey 1987, Lee and Hahn 1988]. Evidence that protein denaturation in higher eukaryotes occurs within the temperature range needed for thermal killing came from DSC and ESR studies [Lepock et al. 1988, 1990, Burgman and Konings 1992]. This denaturation may subsequently lead to protein aggregation [Bensaude et al. 1991, Burgman and Konings 1992, Skowyra et al. 1990, Höll-Neugebauer et al. 1991, Jakob et al. 1993]. Heat-induced protein denaturation and aggregation has been shown to take place throughout the entire cell including the nucleus. Protein aggregation (insolubilization) of nuclear proteins, measured as an increase in the protein content of nuclei, nuclear matrices and chromatin isolated from heated cells has been shown to correlate with thermal killing under a number of conditions [Tomasovic et al. 1978, Roti Roti et al. 1979, 1982, Roti Roti and Winward 1980, Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a, Roti Roti and Laszlo 1988, Borrelli et al. 1992, Stege et al. 1994]. Nuclear protein aggregation is heat dose dependent and is higher when cells are heated in the presence of heat sensitizers like ethanol and procaine [Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a]. The aggregation is lower when cells are heated in the presence of heat protectors e.g. D₂O, glycerol and cycloheximide [Henle and Warters 1982, Kampinga et al. 1989a, Borrelli et al. 1992]. Thermotolerant cells, however, do not always show a reduced formation of nuclear
protein aggregates. Heat-induced thermotolerance in HeLa S3 cells was not [Kampinga et al. 1987, 1989a] or only moderately [Roti Roti and Turkel 1994a] accompanied by a protection against heat-induced protein aggregation. CHO-HA1 TT cells also show no protection [Laszlo 1992b]. On the other hand, Rat-1 TT cells [Stege et al. 1994] and CHO TT cells [Borrelli et al. 1992, 1993] do show resistance against the formation of nuclear protein aggregates. In these studies, the effect of thermotolerance was tested at different time points after the thermotolerance triggering dose, which might account for the observed differences.

A consistent finding in all the cell lines investigated so far is an accelerated recovery from protein aggregates in heat-induced thermotolerant cells. This suggests that the thermotolerance effect on cell killing is anyhow associated with a faster recovery from heat damage. Recently, it was observed that protection against nuclear protein aggregation or enhanced disaggregation may dominate in providing thermotolerance when cells are heated at different temperatures [Borrelli et al. 1993]. At 43°C both processes were shown to be important, whereas protein disaggregation was shown to be the most prominent process in thermotolerance at 45°C. Laszlo [1988a], based on measurements on RNA and protein synthesis, also concluded that thermotolerance is related to ‘better’ repair. The observed initial protection against heat-induced inhibition of RNA as well as protein synthesis, also apparent from his data, appeared to be dependent on the time between the TT trigger and the test dose. This protection as such did however not correlate well with the development and decay of thermotolerance, but seemed related to the elevated synthesis of hsp70.

Since the data from the literature seem contradictory considering the protection against heat-induced nuclear protein aggregation in thermotolerant cells and since it is unclear whether the rates of nuclear protein disaggregation parallel the decay of thermotolerance, we decided to perform a detailed study on the impact of thermotolerance on heat-induced nuclear protein aggregation and subsequent disaggregation in HeLa S3 cells. The latter processes were examined during development and decay of thermotolerance and related to the cellular levels of hsp27, hsp60, hsp70, hsc70 and hsp90.

6.2 MATERIALS AND METHODS

6.2.1 Cell culture, heat treatment and cell survival

HeLa S3 cells were grown in suspension culture in Jokliks modification of minimal essential medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland). Asynchronously, exponentially growing cells with doubling times between 22 and 26 hours were used in all experiments. Hyperthermia was performed in precision waterbaths (± 0.05°C) under continuous gentle shaking. Thermotolerance was induced by a heat treatment of 15 min at 44°C. The level of thermotolerance was
tested using a heat dose of 45 min at 45°C. Cell survival was determined by the use of the cloning technique on soft agar as described earlier [Jorritsma and Konings 1983]. The plating efficiency of untreated HeLa S3 cells was always more than 85%.

6.2.2 Isolation of nuclei and flow cytometry analysis

Relative changes in the protein content of isolated nuclei, as a measure for nuclear protein aggregation were measured according to a slightly modified method of Blair et al. [1979]. Cells were pelleted (5 min at 800 g) and washed 3 times with phosphate (50 mM) buffered saline, followed by washing 3 times in a detergent TX-100 solution (1% TX-100, 0.08 M NaCl, 0.1 M EDTA; pH 7.2) to isolate nuclei. Nuclei, free of major cytoplasmic contaminations, were washed once in TNMP (10 mM Tris-base, 10 mM NaCl, 5 mM MgCl₂ and 0.1 mM phenylmethylsulfonyl-fluoride; pH 7.4) and stained for at least 8 h with 3 µg/ml FITC (fluorescein isothiocyanate) and 35 µg/ml PI (propidium iodide). All procedures were done on ice. 10,000 nuclei were then analyzed on a Becton Dickinson FACS 440 or FACS-STAR flow cytometer. The nuclear protein content relative to the control was determined by computing the mean of the FITC fluorescence distribution of the nuclei from heated cells and dividing it by that of the nuclei isolated from control cells. PI staining was used as a control for possible cell cycle changes during the treatment [Roti Roti et al. 1982, 1986]. From curves representing initial heat-induced nuclear protein aggregation and subsequent disaggregation, the parameter "excess nuclear protein hours" (ENPH) can be calculated [Kampinga et al. 1989a] without using a toxicity threshold [Warters et al. 1986].

6.2.3 Protein gel electrophoresis and immunoblotting

For measurements of cellular hsp levels, cells were resuspended in TNMP and mixed with equal volumes of 2x sample buffer (140 mM Tris, pH 6.8, 2.0 M Glycerol, 200 mM SDS, 10% β-mercaptoethanol and 0.02% Bromphenol blue) and boiled for 5 min prior to electrophoresis. Equal number of cells (1.25 x 10⁵) were electrophoresed through a 12.5% polyacrylamide gel. Immunoblotting was done as described by Towbin et al. [1979] after the electrophoresis. Immunodetection was achieved by incubation of the filters with monoclonal antibodies against hsp27 (SPA 800, Stressgen, Sanbio bv, The Netherlands), hsp60 (SPA 805, Stressgen, Sanbio bv, The Netherlands), hsp70 and hsc70 (SPA 810, SPA 815, SPA 820, Stressgen, Sanbio bv The Netherlands) and hsp89 (a generous gift of Dr. A.C. Wikström, Sweden) followed by reaction with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin. Second antibodies (alkaline phosphatase) and colour reagents were purchased from Pierce Europe B.V. (The Netherlands). Non heated HeLa S3 cells (1.75x10⁴ cells/µl) were used to check the linearity of the response. All values were subsequently related to non-heated control cells using this as a calibration curve. The alkaline phosphatase staining was at least linear to a level of 3.5x10⁴ non-heated HeLa S3 cells.
6.3 RESULTS

HeLa S3 cells were heated at 44°C for 15 min to induce thermotolerance. After this trigger dose the cells were incubated 0 - 30 h at 37°C before a second (test) heat dose of 45 min at 45°C. The triggering heat dose induces a rapid development of thermotolerance in HeLa S3 cells (figure 6.1). The level of thermotolerance decreases gradually after reaching its maximum around 4 h after the trigger. In parallel experiments, cells were sampled for one dimensional electrophoresis and western blotting to determine the total cellular levels of heat shock proteins during development and decay of thermotolerance. These samples were blotted with antibodies against hsp27, hsp60, hsp70, hsc70 and hsp90. In figure 6.2, the cellular levels of these hsp's are depicted. Hsp27 shows a rapid increase followed by a gradual decrease during thermotolerance decay (figure 6.2a). Hsp60 shows a rapid but transient increase (figure 6.2b). The rapid increase of both these hsp's resembles the rapid development of thermotolerance in these cells. The increase of hsp70 and hsc70 is more gradual, reaching a maximum around 16 to 20 h after the trigger (figure 6.2c,d). Thus, levels of these proteins still increase while thermotolerance already decreases (figure 6.1). Hsp90 levels only slightly increased during thermotolerance development and decreased to control levels during the decay of thermotolerance (data not shown).

Next, the effect of development and decay of thermotolerance on heat-induced intranuclear protein aggregation and subsequent disaggregation was studied. Non-thermotolerant HeLa S3 cells heated at 45°C for 45 min showed an increase in the relative FITC fluorescence of nuclei isolated after the heat treatment of about 2.60. During the development and decay of thermotolerance, some protection against the formation of heat-induced intranuclear protein aggregates was found in the TT cells. The effect was maximal at about 10 h after the thermotolerance trigger (figure 6.3a): at this time point, protein aggregation was about 40% less than in non-tolerant cells. To study the effect of thermotolerance on the 'repair' of nuclear protein aggregates, cells were allowed to recover after the 45 min heat treatment at 45°C for 0-6 h at 37°C before nuclei were isolated. The rate of recovery from nuclear protein aggregates was most rapid in cells heated 4/6 h

Figure 6.1 Development and decay of thermotolerance. The surviving fraction of HeLa S3 cells after a test dose of 45 min at 45°C was measured 0 - 30 h after a thermotolerance triggering dose of 15 min at 44°C. The shaded band represents the effect of 45 min at 45°C directly after the thermotolerance triggering dose.

after the trigger dose (figure 6.3b). Plotting the rate of recovery from protein aggregates (slope of disaggregation curves (figure 6.3b)) versus the time after the TT trigger reveals a picture (figure 6.3c) that resembles the development and decay of
thermotolerance at the survival level (figure 6.1) more closely than the effects of thermotolerance on protection against protein aggregation (figure 6.3a).

Next, measurements of initial protection and faster recovery were combined by calculating the area under the increase plus recovery curves. Plotting this parameter, previously termed 'excess nuclear protein hours' (ENPH) [Kampinga et al. 1989a] as a function of time after the TT trigger (figure 6.3d) shows a pattern that very closely resembled the pattern for survival (figure 6.1).

When a correlation plot was made between the surviving fraction and the amount of protein aggregates measured immediately after the test dose (figure 6.4), it can be seen that there is a significant (P<0.05) but weak ($r^2=0.604$) correlation between these parameters. The rates of disaggregation showed a better correlation ($r^2=0.797$) with the surviving fraction (figure 6.5). Yet, the best correlation ($r^2=0.882$) with cell survival was found for the ENPH parameter (figure 6.6).
Figure 6.2 Cellular hsp levels (arb. units) during development and decay of thermotolerance. HeLa S3 cells were heated (15 min at 44°C) and samples were taken after 0 - 30 h at 37°C to perform Western analysis for total cellular hsp levels.
Western blots were scanned and mean values (± SEM) are depicted (n=2-3). Western blots from typical experiments are shown. (a) hsp27; (b) hsp60; (c) hsp70; (d) hsc70.
Figure 6.3 Heat-induced nuclear protein aggregation and disaggregation after a test dose of 45 min at 45°C during development and decay of thermotolerance. (a) Formation of nuclear protein aggregates. The relative FITC fluorescence of HeLa S3 cells after a test dose of 45 min at 45°C was measured 0 - 30 h after a thermotolerance triggering dose of 15 min at 44°C. Mean values (± SEM) of 4 experiments are given. (* recalculated from Kampinga et al. 1992). The shaded band represents the effect of 45 min at 45°C directly after the thermotolerance triggering dose. (b) Disaggregation of heat-induced nuclear protein aggregates. The ratio of relative FITC fluorescence was measured 0 - 6 h (37°C) after a test dose of 45 min at 45°C given 0 - 30 h after a thermotolerance triggering dose.
of 15 min at 44°C. (Initial increase is set to 1.0 for comparison of the rate of disaggregation). The numbers indicate the hours after the thermotolerance trigger at which the test dose is given. (* recalculated from Kampinga et al. 1992). (c) Rates of disaggregation. The rate of disaggregation is determined as the slope of the disaggregation curves of figure 6.3b and plotted against the time (0 - 30 h) after the thermotolerance trigger of 15 min at 44°C. (d) Excess nuclear protein hours (ENPH). ENPH is calculated from the combined aggregation and disaggregation curves as described in Materials and methods and plotted against the time (0 - 30 h) after the thermotolerance trigger of 15 min at 44°C.
Figure 6.4 Correlation between heat-induced nuclear protein aggregates formed and cell survival. The surviving fraction of HeLa S3 cells (data from figure 6.1) is plotted against the relative FITC fluorescence measured immediately after a test dose of 45 min at 45°C during development and decay of thermotolerance (data from figure 6.3a).

Figure 6.5 Correlation between the rate of protein disaggregation after hyperthermia and cell survival. The surviving fraction of HeLa S3 cells (data from figure 6.1) is plotted against the reciprocal of the rate of disaggregation of heat-induced nuclear protein aggregates after a test dose of 45 min at 45°C test dose during development and decay of thermotolerance (data from figure 6.3c).
Figure 6.6. Correlation between excess nuclear protein hours (ENPH) and cell survival. The surviving fraction of HeLa S3 cells (data from figure 6.1) is plotted against ENPH after a test dose of 45 min at 45°C during development and decay of thermotolerance (data from figure 6.3d).

6.4 DISCUSSION

6.4.1 Thermotolerance: protection against nuclear protein aggregates

The controversial effects of thermotolerance on the formation of heat-induced protein aggregates may be due to differences in thermotolerance induction dose, different time intervals between TT trigger dose and test dose and/or the test doses used. Kampinga et al. [1987, 1989a, 1992] found no significant effect of thermotolerance on initial nuclear protein aggregation 5/6 h after a trigger of 15 min at 44°C. Increasing the test dose (h at 45°C) leads to reduced initial protein aggregation in TT cells 5 h, but not at 24 h after a TT trigger of 15 min at 45°C or 30 min at 45°C [Roti Roti and Turkel 1994a]. Borrelli et al. [1993] showed that protection in TT CHO cells was most prominent at 43°C instead of 45°C. These authors concluded that at 43°C but not at 45°C both protection and accelerated disaggregation occurring during heating together contribute to a drastic reduction in the protein aggregation measured at the end of the heat treatment. During heating at 45°C no disaggregation could take place and less, albeit significant, protection was found.

However, in CHO cells, less initial damage was found 3 h after the TT trigger [Borrelli et al. 1992], but no protection was found when CHO-HA1 cells were heated 15 h after the trigger dose [Laszlo 1992b]. So, also in CHO cells the presence/absence of a thermotolerance effect on protection against heat-induced nuclear protein aggregation may also be dependent on the time interval between the trigger dose and test dose like was found in our hands for HeLa S3 cells (figures 6.3...
and 6.4). As shown in figures 6.3 and 6.4, the protection against the formation of nuclear protein aggregates did however not mirror the development and decay of thermotolerance. The heat-induced elevations of hsp70/hsc70 levels (figure 6.2c) showed the same profile as protection against the formation of heat-induced intranuclear protein aggregates (figure 6.3a): a significant correlation was found between these two parameters ($r^2 = 0.80; p < 0.05$). This suggests that hsp70 or and hsc70, is/are involved in protection against heat-induced intranuclear protein aggregation, as observed previously for hsp70 [Stege et al. 1994]. As being translocated to the nucleus upon heat shock [Welch and Feramisco 1984, Welch and Mizzen 1988, Hayashi et al. 1991] a higher level of induced (free) hsp70/hsc70 may exert its action as molecular chaperone in the nucleus by binding to partially denatured nuclear proteins, attenuating their (further) aggregation. HeLa S3 cells, unlike rodent cells, already contain substantial levels of hsp70, and only a major increase in the level of this protein (10-15 h after the TT trigger) may lead to protection against the formation of nuclear protein aggregates.

So, differences in hsp70 levels (endogenous and induced) in thermotolerant cells (at different time points after the trigger) possibly together with different test doses used may be responsible for the apparently conflicting reports on the protective effects of thermotolerance against heat-induced nuclear protein aggregation [Kampinga et al. 1987, 1989a, Laszlo 1992b, Borrelli et al. 1992, Stege et al. 1994].

Hsp90 showed only a slight increase after this trigger dose. Also for hsp90 involvement in heat resistance has been shown. Reduction in hsp90 levels resulted in increased heat sensitivity [Bansal et al. 1991] and hsp90 overexpression resulted in heat resistance [Yahara et al. 1986]. As translocated to the nucleus [Akner et al. 1992] (data not shown) it might have contributed to the observed protection against nuclear protein aggregation in TT cells, which would be in accordance with its suggested chaperone activity observed in cell free approaches [Wiech et al. 1992].

### 6.4.2 Thermotolerance: enhanced disaggregation of nuclear protein aggregates

Our experiments with thermotolerant cells show that always a faster disaggregation of the protein aggregates was found, which confirms earlier observations [Kampinga et al. 1987, 1989a, 1992, Laszlo 1992b, Borrelli et al. 1992, Stege et al. 1994]. Better 'repair' of heat-induced damage in thermotolerant cells was also found for nucleolar morphology [Welch and Mizzen 1988], RNA and protein synthesis [Laszlo 1988a, 1992b] and may be a more general phenomenon for thermotolerant cells. Inhibition of protein synthesis during the development of thermotolerance abolished the faster disaggregation [Borrelli et al. 1993], indicating that newly synthesized proteins are necessary for this process. In the current study, the rate of the disaggregation process seems to parallel the development and to somewhat less extent the decay of thermotolerance.
Hsp27 levels paralleled the rates of recovery from the heat-induced aggregates (correlation analysis: $r^2 = 0.94; p < 0.05$). A role for elevated hsp27 levels in recovery from nuclear protein aggregates is also suggestive from data using hsp27 overexpressing, heat resistant, rodent cells [Landry et al. 1989, Kampinga et al. 1994]. Although studies using cell free systems recently demonstrated a chaperone-like activity of hsp27 as an attenuator of protein aggregation [Jakob et al. 1993, Merck et al. 1993], such an activity is not suggestive from our in situ studies. Cellular hsp27 levels did not parallel protection by the thermotolerant state against heat-induced nuclear protein aggregation (this study) and hsp27 overexpressing cells did not show protection against that process [Kampinga et al. 1994]. Whether hsp27 is directly involved in the recovery process or indirectly, e.g. via protective mechanisms elsewhere in the cell [Lavoie et al. 1993], remains to be elucidated. Hsp27 has been found to translocate to the nucleus after heating and to aggregate herein [Arrigo et al. 1988]. Such association with the bulk of nuclear protein aggregates may be a functional first step in the pathway of protein disaggregation. So, (parts of) the reported protective action by which elevated hsp27 expression contributes to heat-induced thermotolerance (Landry et al. 1991) may be via its involvement in the process of (nuclear) protein disaggregation.

As hsp70 levels do not correlate with the accelerated disaggregation seen in TT cells ($r^2 = 0.60; p > 0.05$), our results confirm and extent upon the observation that hsp70 is not involved in enhanced recovery from nuclear heat damage [Stege et al. 1994] unlike previously suggested [Pelham 1984, Welch and Mizzen 1988]. For hsc70 the situation is unclear. Hsc70 has been shown to facilitate the uncoating and release of clathrin triskelions from clathrin-coated vesicles [Ungewickel et al. 1985, Chappell et al. 1986, DeLuca-Flaherty et al. 1990]. Under stress conditions, an involvement in disaggregation of nuclear protein aggregates has been suggested [Laszlo 1992b], since mutant CHO-HA1 cells (3012) that overexpress hsc70 show facilitated nuclear protein disaggregation. In the current study, no correlation between the more rapid nuclear protein disaggregation and (elevated) cellular levels of hsc70 was observed. The reason for this difference is yet unclear.

This is the first study showing a correlation between increased cellular hsp60 levels and the development of thermotolerance. However, hsp60 levels seem not required to sustain thermotolerance, since these have returned to background values at time points where still substantial thermotolerance was observed. Hsp60, localized in the mitochondria, might be involved in the protection against mitochondrial protein damage. The role, if any, of hsp60 in nuclear protein aggregation/disaggregation remains unclear.

In conclusion, the results of the current study show that thermotolerance is related to both protection against as well as better recovery of heat-induced nuclear protein damage. Measurements of cellular hsp levels suggest that hsp’s play a role in
thermotolerance either by protection against heat-induced intranuclear protein aggregates (hsp70) or by enabling a faster disaggregation of these aggregates (hsp27). The total cellular levels of none of the individual hsp’s completely correlate with development and decay of thermotolerance indicating that overexpression of any of these hsp’s alone does not determine the level of thermotolerance. In HeLa S3 cells, enhanced disaggregation paralleled the development of thermotolerance. This seems in agreement with the observations of Borrelli et al. [1993], indicating that the tolerant state in the first hours after the trigger dose is predominantly determined by faster recovery. Increasing the time between the trigger and test dose might shift it towards protection against protein aggregation as the main defense pathway to survive a heat treatment.

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