Hyperthermia and protein aggregation
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THERMAL PROTEIN DENATURATION AND PROTEIN AGGREGATION IN CELLS MADE THERMOTOLERANT BY VARIOUS CHEMICALS: ROLE OF HEAT SHOCK PROTEINS

This chapter is submitted for publication
ABSTRACT

Thermotolerance (TT) induced by sodium-arsenite (A-TT: 100 µM, 1 h 37°C), ethanol (E-TT: 6% (v/v), 25 min 37°C) and diamide (D-TT: 300 µM, 1 h 37°C) was compared to heat-induced thermotolerance (H-TT: 15 min 44°C) using HeLa S3 cells. All four pre-treatments lead to comparable levels of thermotolerance and also induce resistance to arsenite-, ethanol-, and diamide-induced toxicity (clonogenic ability). Stress-induced expression of the major heat shock proteins (hsp27, hsc70, hsp70, and hsp90) was generally the highest in H-TT cells and the lowest in A-TT cells. Interestingly, the four types of TT cells showed distinct differences in certain aspects of resistance against thermal protein damage. Thermal protein denaturation and aggregation determined in isolated cellular membrane fractions was found to be attenuated when they were isolated from H-TT and A-TT cells but not when isolated from E-TT and D-TT cells. The heat resistance in the proteins of the membrane fraction corresponded with elevated levels of hsp70 associated with the isolated membrane fractions. In the nuclear fraction, only marginal (not significant) attenuation of the formation of protein aggregates (as determined by TX-100 (in)solubility) was observed. However, the post-heat recovery from heat-induced protein aggregation in the nucleus was faster in H-TT, E-TT and D-TT cells, but not in A-TT cells. Despite the fact that elevated levels of hsp27, hsc70 and hsp70 were found in the TX-100 insoluble nuclear fraction derived from all TT cells, no correlation was found with the degree of resistance in terms of the accelerated recovery from nuclear protein aggregation. The only correlation between accelerated recovery from nuclear protein aggregates was that with total cellular levels of hsp27.

The data indicate that heat-induced loss of clonogenic ability may be a multitarget rather than single target event. A threshold of damage may exist in cells after exposure to heat; multiple sets of proteins in (different compartments of) the cell need to be damaged before this threshold is exceeded and the cell dies. As a consequence, stabilization of only one of these sets of proteins is already sufficient to render cells thermotolerant at the clonogenic level.
7.1 INTRODUCTION

Although the exact pathways leading to hyperthermic cell killing have not yet been elucidated, it seems that protein damage -denaturation and (subsequent) aggregation- is a key step in this process [Lepock et al. 1988, 1990, 1993, Bensaude et al. 1991, Lepock and Kruuv 1992, Burgman and Konings 1992, Burgman et al. 1993, Kampinga 1993]. During rise of temperature, the kinetic energy of all cellular molecules increases; in other words, the absorbed energy is diffusely distributed over the cell. As a consequence, thermal protein denaturation and aggregation may occur at any place throughout the entire cell where thermolabile proteins are found. Recently, Lepock and coworkers [1993] showed that such proteins can be indeed found in all cellular fractions investigated (nuclei, microsomes, mitochondria).

The transient resistance against thermal killing (clonogenic ability) as it can be induced by prior heating (heat-induced thermotolerance: H-TT) was shown to correlate with a resistance against thermal protein damage, reflected in less induction and/or better recovery from thermal protein damage in situ [Kampinga et al. 1987, 1989a, Lepock et al. 1990, 1993, Bensaude et al. 1991, Lepock and Kruuv 1992, Burgman and Konings 1992, Burgman et al. 1993, Kampinga 1993,]. It has also been suggested [Lee and Hahn 1988] that expression of H-TT is the result of a response of cells to the thermal denaturation/aggregation caused by the TT triggering treatment. In addition, thermal damage to proteins has been proposed as the trigger for activation of heat shock genes [Hightower 1980, Ananthan et al. 1986, Edington et al. 1989] resulting in elevated levels of heat shock proteins (hsp’s) in the cell that result in the acquisition of thermotolerance at the clonogenic level. As such, in mammalian cells, elevated hsp70 (Hahn and Li 1982, Li et al. 1991), hsp27 [Landry and Chrétien 1983, Landry et al. 1989], and recently also hsp40 [Ohtsuka et al. 1990, Hattori et al. 1993] have been related to the development in thermotolerance.

Thermotolerance can also be induced by prior treatment of cells with a variety of chemicals followed by a drug-free period before the heat challenge [Kampinga et al. 1992]. Unlike H-TT, however, the resistance of proteins that accompanies these types of thermotolerance seems not to be distributed uniformly throughout the cell. It was e.g. found that cells made tolerant from prior sodium arsenite treatment (A-TT) show no resistance against heat-induced nuclear protein aggregation [Kampinga et al. 1992] whereas they do show resistance against thermal denaturation and aggregation of membrane proteins [Burgman et al. 1993].

Considering the multiple lesions that are induced by heat throughout the entire cell, the question can be asked whether heat killing is a single or multitarget process and whether a threshold of damage may exist before cells die from a heat injury. In the latter case, stabilization of only one target would increase the threshold and make cells tolerant at the clonogenic level. The differences in intracellular distributions of the various hsp’s and their redistributions after heat is in favour of the
idea that heat damages proteins molecules throughout the cell and subsequently protect the previously damaged proteins become resistant against a subsequent heat insult. It is yet unclear if the character of protein damage or/and the specific site where the damage occurs in the cell is selective for the action of specific hsp’s. Furthermore, there are indications that some hsp’s are involved in protection against thermal protein damage, whereas other hsp’s play a role in the (accelerated) recovery from that damage [Kampinga 1993].

In the current study, cells that were made thermotolerant by prior heating (H-TT) were compared to those made thermotolerant by pre-treatment with sodium-arsenite (A-TT), ethanol (E-TT), or diamide (D-TT), and to non-pre-treated control cells. These chemicals are well-known for their ability to induce thermotolerance. Unlike heat they are likely to be more specific in the type of protein damage they inflict or/and in the intracellular compartment they induce (most) protein damage. As such they may cause a specific induction of certain hsp’s or trigger specific reallocations of hsp’s and lead to resistance of protein damage in restricted compartments only. First, H-TT, A-TT, E-TT, and D-TT cells were tested for their resistance against the toxic action of heat, arsenite, ethanol, and diamide (clonogenic ability). Furthermore, resistance against thermal protein damage was tested in two different cellular subfractions. Electron spin resonance and thermal gel analysis were used to measure protein denaturation and aggregation in crude fractions containing cellular membranes. Nuclear protein damage (nuclear protein (dis)aggregation) was measured by analyzing the relative amount of TX-100 insoluble nuclear proteins using a flow-cytometric approach. The presence/absence of resistance in these two fractions isolated from the various types of TT cells was subsequently compared to the relative expression of hsp27, hsp60, hsp70, hsc70, and hsp90 and their association with the subcellular fractions.

7.2 MATERIAL AND METHODS

7.2.1 Cells and culture conditions
HeLa S3 cells (ATCC no. CCL 2.2) were grown in suspension culture in Joklik’s modification of minimal essential medium supplemented with 10% foetal bovine serum. Asynchronously, exponentially growing cells (doubling time 22-26 h) with a trypan blue exclusion of over 95% were used in all experiments.

7.2.2 Incubation conditions
Hyperthermia was performed in precision waterbaths (± 0.1°C) under continuous gentle shaking. To induce thermotolerance, cells were either heated for 15 min at
44°C (heat induced tolerance: H-TT), incubated for 1 h at 37°C with 100 µM sodium arsenite (arsenite induced tolerance: A-TT), for 1 h with 300 µM diamide (diamide induced tolerance: D-TT), or for 25 min at 37°C with 6% (v/v) ethanol (ethanol induced tolerance: E-TT) and incubated for 4 (E-TT) or 5 (H-TT, A-TT and D-TT) h at 37°C in absence of the drug to allow for tolerance development.

### 7.2.3 Determination of cell survival

The clonogenic ability of the cells was tested as previously described using the soft agar technique [Kampinga et al. 1985]. Colonies (containing more than 50 cells) were counted after 10-14 days. Plating efficiency of untreated cells was more than 80%.

### 7.2.4 Isolation of the sub-cellular fractions

The particulate fraction (PF) was isolated as previously described [Burgman and Konings 1992]. In short, cells were harvested, washed with phosphate-buffered saline (PBS) and swollen in a hypotonic buffer (TNM: 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1.5 mM MgCl₂) for 20 min at 0°C. After Dounce homogenization, the nuclei were spun down (10 min at 1000 g) and the supernatant was centrifuged again for 60 min at 100000 g to yield the particulate fraction (PF) as a pellet. Further subfractionation showed that this PF is representative for mitochondrial/lysosomal and plasmamembrane proteins [Burgman et al. manuscript in preparation].

To determine nuclear protein (dis)aggregation, HeLa cells were harvested, washed twice with PBS and subsequently two times with a TX-100 solution (1% Triton X-100; 0.08 M NaCl; 0.01 M EDTA; pH=7.2) and once in TMNP (10 mM Tris-base; 10 mM NaCl; 5 mM MgCl₂; 0.1 mM phenylmethylsulfonyl-fluoride; pH=7.4). The final TX-insoluble pellet (containing morphologically clean nuclei free of cytoplasmic contamination) was resuspended in 0.4 ml TNMP. The nuclei were either stained with 0.1 ml FITC (3 µg/ml final concentration) and 0.5 ml PI (35 µg/ml final concentration) to measure the relative nuclear protein content flow cytometrically or they were pelleted once more and run for SDS-PAGE/Western analysis.

All isolation procedures were performed at 0-4°C.

### 7.2.5 Electron Spin Resonance (ESR)

For the ESR measurements, the sulfhydryl groups of the proteins in the various membrane fractions were labelled with 4-maleimido-tempo as previously described [Burgman and Konings 1992, Burgman et al. 1993]. ESR spectra were recorded on a Varian E-4 spectrometer with a nitrogen flow heating device. Recorder settings were: scan range 200 g (except figure 7.1; 100 g), scan time 4 min, modulation amplitude 1.0 g, time constant 0.3 sec, and microwave power 10 mW. The gain setting depended on the concentration of the sample but was usually between 5x10² and 10³. The samples were heated at a rate of approx. 1°C/min and partial ESR spectra (first
derivative) were recorded every 2-3°C between 20 and 70°C. From each spectrum the ratio (R) between the peaks representing weakly (W) and strongly (S) immobilized spin label was determined and transition points were determined (see Burgman and Konings [1992] for details). For each sample, $10^8$ cells were used.

7.2.6 Thermal Gel Analysis (TGA)

TGA was performed as previously described [Burgman and Konings 1992]. In short, aliquots of 25 µl, containing the PF of 5x10^6 cells in PBS, were heated at a rate of 1°C/min. At different temperatures samples were withdrawn an prepared for non reducing PAA-SDS gel electrophoresis. After electrophoresis, the gels were stained with Coomassie Brilliant Blue and the intensity of the protein bands was determined densitometrically. The intensity was normalized relative to the intensity of unheated samples (100%) and the intensity of samples heated to over 60°C (0%). The relative intensity was plotted against the temperature, and from these plots the temperature at which the relative intensity was reduced to 50% (thermal midpoint) was determined.

7.2.7 Flow cytometric determination of nuclear protein content

10,000 nuclei were analyzed on a Becton Dickinson FACS IV flow cytometer/sorter according to the method of Blair et al. [1979]. The nuclear protein content relative to control was determined by computing the mean of the FITC fluorescence distribution of nuclei from heated cells and dividing it by that of the nuclei isolated from control cells. The actual protein mass on a per nucleus basis using this isolation technique is about 46 pg protein in a unheated control cell [Kampinga et al. 1989a]. Measurement were performed either immediately after heating the cells (nuclear protein aggregation) or 1-5 h thereafter (disaggregation).

7.2.8 Protein gel electrophoresis and immunoblotting

Cells, particulate fractions (PF), or isolated nuclei were dissolved in either TMNP (nuclei) or PBS (cells and PF). Cellular and nuclear samples were DNase I digested (1 mg/ml DNase I for 15 min at 37°C). All samples were mixed with equal volumes of 2x sample buffer (125 mM Tris, pH 6.8, 2.7 M Glycerol, 1.4 mM β-mercaptoethanol, 90 mM SDS, and 0.72 mM bromophenolblue) and boiled for 5 min prior to electrophoresis. The samples (10-60 µl) were loaded and electrophoresed through a 4.8% stacking into a 10% polyacrylamide gel. Equal numbers of particles were loaded in the case of cells and nuclei. For the PF, loading was done on basis of equal protein determined by the method of Lowry et al. [1951].

Immunodetection of proteins immobilized on nitrocellulose membranes was performed as described previously [Hahn and Li 1982, Kampinga et al. 1992]. Briefly, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose filters (BioRad) and probed with antibodies raised against specific hsp’s. The following antibodies were used: anti-hsp27 (StressGen SPA-800), anti-hsp60 (StressGen SPA-
Protein denaturation, thermotolerance, and hsp

805), anti-hsp70 (StressGen SPA-810, also referred to as C92), anti-hsc70 (StressGen SPA-815), and anti-hsp89 (a generous gift of Dr. A.C. Wikström, Sweden). Immune complexes were detected using alkaline phosphatase techniques using 4-BCIP/NBT in substrate buffer (100 mM Tris pH=9.5; 100 mM NaCl; 5 mM MgCl₂).

7.3 RESULTS

In figure 7.1, the effect of heat-, arsenite-, ethanol, and diamide pretreatment on the cytotoxic action of heat-, arsenite-, ethanol, and diamide is depicted. All data are corrected for cell killing caused by the pre-treatments. The percentage cell killing by these triggering treatments was below 20% with the exception of the diamide pre-treatment killing 45% of the cells. All pre-treatments were chosen such that they lead to comparable levels of thermotolerance (figure 7.1a). Also, resistance against the toxic action of the tested chemicals could be found after all these pre-treatments (figure 7.1b-d). Although there is some scatter, the levels of resistance show no general trends for specific cross-tolerance for the various types of inducers. So, all TT cells are indistinguishable when tested at the level of clonogenic resistance against heat and drug-toxicity.

Unlike their comparable levels of resistance at the clonogenic level, the pattern of hsp-expression varied for the 4 pretreatments. As can be seen in figure 7.2, the pre-heat treatment of cells leads to the largest increase in all levels of hsp tested, whereas the arsenite pre-treatment caused the most moderate increase in total cellular hsp levels, hsp27 in particular. Similar observations were made for hsp60 (not shown). For all pretreatments, increases in hsp70 and hsp27 levels were the highest (figure 7.2a,b). Only moderate increases in cellular levels of hsc70 (figure 7.2c), hsp90 (figure 7.2d), and hsp60 (not shown) were detected.

In previous studies on HeLa S3 cells, it was shown that membrane proteins in H-TT cells were resistant against thermal denaturation as measured by ESR (see Burgman and Konings [1992] for details). These studies were done by in vitro heating (1°C/min heating rate) of isolated particulate fractions (PF) containing the
Figure 7.1 Stress-induced resistance against the toxicity (clonogenic ability) of (a) heat, (b) sodium-arsenite, (c) diamide, and (d) ethanol. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT (★)), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT (●)), diamide (1 h 300 µM + 5 h 37°C: D-TT (♦)), or ethanol (25 min 6% + 4 h 37°C: E-TT (♦)) or left untreated (non-TT (○)) before exposure to heat (45°C: a) or 1 h incubations with graded concentrations of sodium arsenite (b), diamide (c), or ethanol (d). The clonogenic ability of the cells was assayed using the soft agar technique [Kampinga et al. 1985] and the data are corrected for the toxicity of the tolerance inducing pretreatments. Mean survival data (± SEM) are given from 2-3 independent experiments.

plasmamembrane, mitochondria, lysosomes, and microsomes (but no nuclear membranes). Three transition points (T_A, T_B, and T_C) were found of which T_B and T_C were irreversible in vitro and (thus) interpreted to be due to protein denaturation (for actual ESR profiles and their interpretations see Burgman and Konings [1992]). These transition temperatures (T_B ≈ 47°C; T_C = 57°C) for denaturation cannot be taken as absolute values since "thermal dose" is a function of both temperature and time at that temperature. Using the Arrhenius relationship for heat killing for temperatures above 42.5°C [Westra and Dewey 1971], it can be
Protein denaturation, thermotolerance, and hsp

Figure 7.2 Stress-induced elevation in the total cellular levels of hsp27 (a), hsp70 (b), hsc70 (c), and hsp90 (d). HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT), diamide (1 h 300 µM + 5 h 37°C: D-TT), or ethanol (25 min 6% + 4 h 37°C: E-TT) or left untreated (non-TT). The various cells were processed on SDS-PAGE and immunoblotted with specific antibodies, recognizing hsp27, hsp70, hsc70, and hsp90 (see material and methods for details). Mean values (± SEM) are given from 3 independent experiments.

calculated that heating up to 47°C (using 1°C/min heating rates) is roughly equivalent to a 30 min 43°C heat treatment. Such a treatment kills less than 25% of the HeLa S3 cells used in this study (data not shown). To investigate with more precision which membranes were or were not protected from thermal denaturation in H-TT cells, the PF was further sub-fractionated into a plasmamembrane fraction, a mitochondrial/lysosomal fraction, and a microsomal fraction [Burgman et al. manuscript in preparation]. The profile for denaturation in microsomal fraction was found to deviate from the others, but the transitions in the PF closely resembled those obtained in the isolated mitochondria/lysosomal and plasmamembrane fraction [Burgman et al. in preparation]. For practical reasons (about 2x10^9 cells are required
to yield enough pure sub-fractionated membranes to carry out one ESR scan), the experiments comparing the various chemically induced TT cells were done using the PF. The absence/presence of resistance in these fractions thus needs to be interpreted with some caution in terms of specific “targets” involved. However, since they represent the majority of the total PF, the mitochondrial/lysosomal fraction will mainly determine the outcome of the PF data. As shown in figure 7.3, the results with cells made TT by the various chemicals were rather surprising. As found before [Burgman et al. 1993], the PF proteins in the A-TT cells were resistant against thermal denaturation like those isolated from H-TT cells. For both H-TT and A-TT, higher temperatures were found for $T_B$ (figure 7.3a) and for $T_C$ (not shown), while $T_A$ was not affected by any of the pretreatments used (data not shown). However, in D-TT and E-TT cells, having the same heat resistance at the cell survival level (figure 7.1a), no resistance of PF proteins against thermal denaturation were found (figure 7.3a). Next, protein aggregation of PF proteins was measured using non-reducing SDS-PAGE. By heating isolated PF fractions at $1^\circ\text{C/min}$ (like done for ESR), this method detects the heat-induced formation of protein aggregates via S-S bond formation [Burgman and Konings 1992]. Quantification of these gels occurs on the basis of disappearance of some thermolabile proteins from the gel lanes. Comparison between TT and non-TT cells was made by taking the temperature at which 50% of the thermolabile proteins were aggregated (thermal midpoint: for details on the method: see Burgman and Konings [1992]). As shown in figure 7.3b, like with the ESR technique, resistance of PF proteins was found in H-TT and A-TT cells, but not in E-TT cells. This method could not be applied to D-TT cells possibly due to S-S based protein crosslinking by residual diamide.

When hsp levels were measured in (unheated) PF isolated from the various cells, no hsp27 and hsp90 were detected (data not shown) consistent with earlier data on the cellular (re)allocation of these hsp’s at $37^\circ\text{C}$ and after heating [Kampinga et al. 1994]. No significant differences in the levels of hsp60 were found between all samples (figure 7.3c). Hsc70 was elevated in all TT cells compared to non-TT cells. However, no differences were found between hsc70 levels in the PF for the various types of TT. Interestingly, compared to hsp70 levels in PF from non-TT cells, these hsp70 levels were significantly elevated in PF from H-TT and A-TT cells but not in the PF isolated from E-TT and D-TT cells. As can be read from the densitometric quantification of this blot (figure 7.3d), the hsp70 levels in the PF nicely coincide with the observed absence/presence of resistance against thermal protein denaturation (figure 7.3a,b).

In another set of experiments, heat-induced nuclear protein aggregation and subsequent disaggregation was measured in the various TT and non-TT cells. Insolubilization of various cellular proteins has been reported as a consequence of a heat treatment of cells. A higher recovery of nuclear DNA polymerase $\alpha$ and $\beta$
Figure 7.3 Comparison of heat- and chemically induced thermotolerance: resistance of the membrane fraction. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT), diamide (1 h 300 µM + 5 h 37°C: D-TT), or ethanol (25 min 6% + 4 h 37°C: E-TT) or left untreated (non-TT). Subsequently, the particulate fraction from these cells was isolated and thermal protein denaturation (panel a (ESR: $\Delta T_b$ between TT and non-TT controls)) and aggregation (panel b (TGA: $\Delta$Thermal midpoint between TT and non-TT controls)) was determined. Also, these isolated fractions were processed on SDS-PAGE and immunoblotted with specific antibodies, recognizing hsp27, hsp60, hsp70, hsc70, and hsp90 (see material and methods for details). In panel c, a typical immunoblot is depicted showing hsp 60, hsp70, and hsc70. In panel d, the quantification of hsp70 levels in the PF, as measured by densitometric analysis, is given. Hsp27 and hsp90 could not be detected in any of the fractions (data not shown).

activity was found in nuclei isolated from heated cells [Kampinga et al. 1985]. Also, a decrease in solubility of the nuclear (proto)oncogene products (myc, myb, and p53 families), RNA polymerases, p68 kinase, (transfected) β-galactosidase and (transfected) luciferase occurs in heat-shocked cells [Evan and Hancock 1985, Littlewood et al. 1987, Luscher and Eisenman 1988, Nguyen et al. 1989, Fisher et al. 1989, Dubois et al. 1991]. This insolubilization of these identified nuclear proteins may have to be
considered as a part of the widely observed overall increase in the protein mass of nuclear structures when isolated from heated cells [Roti Roti and Winward 1978, Tomasovic et al. 1978, Kampinga et al. 1985, 1987, 1989a, 1992, Warters et al. 1986, Borrelli et al. 1992, Kampinga 1993, Chu et al. 1993, Stege et al. 1994]. The heat-induced increase in the overall protein mass of isolated nuclei (reduced TX-100 solubility) was shown to be predominantly (≥75%) due to a reduced loss of nuclear proteins during the nuclear isolation [Kampinga et al. 1985, Kampinga 1993, Chu et al. 1993]. The reduced solubility is therefore interpreted as due to the formation of nuclear protein aggregates as a consequence of thermal protein denaturation. The increase in protein mass of isolated nuclei was used as an end-point to assess the extent of protein denaturation/aggregation induced by heat in the various TT cells; also the ability of cells to recover from such aggregates (allowing recovery of the cells at 37°C before isolating the nuclei) was studied (figure 7.4). As found before [Kampinga et al. 1987, 1989a, 1992], in HeLa S3 cells made thermotolerant by prior heating the initial heat (60 min 45°C)-induced protein aggregation is only marginally (not significantly) reduced as compared to that in non-TT cells. The same was true for cells made TT by prior diamide or ethanol treatment. For A-TT cells initial aggregation, if anything, was even slightly higher than in control, non-TT cells (figure 7.4a,b). However, in H-TT HeLa S3 cells resistance at the nuclear level is clearly expressed in terms of an enhanced rate of recovery from nuclear protein aggregates [Kampinga et al. 1987, 1989a, 1992] (figure 7.4a,c). Also in D-TT and E-TT cells, but not in A-TT cells, these recovery rates were found to be more rapid than in non-TT cells (figure 7.4a,c). In previous studies [Kampinga et al. 1989a, Stege et al. 1994], it was demonstrated that the best way to correlate nuclear heat damage with thermal killing is to take the integral under the curve that represents the increase and subsequent recovery. Using this parameter (Area Under the Curve: AUC (figure 7.4d)), it is clear that “nuclear resistance” (in terms of smaller AUC) is found for H-TT, E-TT and D-TT cells, but not for A-TT cells.

Finally, TX-100 insoluble (nuclear) hsp levels were determined before and after heating the cells. No significant levels of hsp60 could be detected in the TX-100 insoluble fraction; hsp90 was present in the TX-100 fraction at low abundance after heating but not before. For the other hsp’s tested, the levels in the TX-100 fractions were a) higher in fractions from unheated TT cells compared to the TX-100 insoluble fraction isolated from non-TT cells and b) elevated after heating all cells. Yet, no relation was found for the amount of any of the TX-100 insoluble hsp’s with the (dis)aggregation of the bulk of the heat-induced insolubilized proteins (data not shown).
Figure 7.4 Comparison of heat- and chemically induced thermotolerance: resistance of the nuclear fraction. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT (•)), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT (♦)), diamide (1 h 300 µM + 5 h 37°C: D-TT ( ♦)), or ethanol (25 min 6% + 4 h 37°C: E-TT (♦)), or left untreated (non-TT (o)) before exposure to heat (60 min 45°C). (a) Nuclei (TX-100 insoluble material) were isolated either immediately after heating the cells or after allowing the cells to recover 1-5 h at 37°C. The TX-100 insoluble fraction was stained with FITC and PI and analyzed flow cytometrically (see material and methods). The FITC signals of nuclei, relative to that of nuclei isolated from of unheated non-TT control cells are given. Mean values (± SEM) are given from at least 3 independent experiments. (b) Initial aggregation (replotted from figure 7.4a). (c) Rates of recovery from protein aggregates (calculated from figure 7.4a by regression analysis). (d) The area under the increase plus recovery curve (AUC) from figure 7.4a was calculated up to 5 h post heating.
7.4 DISCUSSION

7.4.1 Cross-resistance and site-specific tolerance concept

The data presented here show that (for the treatment protocols used) the various types of TT inducers bring about heat- and drug resistance (clonogenic ability) in an indistinguishable manner (figure 7.1). Although the exact mode(s) of cell killing are unknown for both the chemicals used and for heat, the observed "cross-tolerance" [Lee and Hahn 1988, Freeman and Meredith 1989, Kampinga et al. 1992] (figure 7.1) could indicate that all thermotolerant cells have acquired resistance to heat and the chemicals (in terms of cell killing) by a similar mechanism: resistance of the same critical, most sensitive "target(s)". Nevertheless, the expression of resistance against thermally induced protein damage (denaturation and aggregation) differs amongst the various inducers (figures 7.3 and 7.4). When heat was used to trigger TT, thermal resistance was found for proteins of both the membrane and nuclear fraction tested. For TT induced by arsenite, membrane proteins but not nuclear proteins showed resistance against thermal protein denaturation/aggregation as already published before [Kampinga et al. 1992, Burgman et al. 1993]. For TT induced by ethanol and diamide, no resistance was found for membrane proteins (ESR/TGA) whereas these TT types did show enhanced rates of recovery from heat-induced nuclear protein aggregates [Burgman et al. 1993, this report]. A simplistic explanation for these observations could be that neither of the parameters measured is of importance in relation to thermal killing or not sensitive enough for a specific subpopulation of (relevant) proteins. Although this possibility cannot be ruled out completely, we favour the assumption that different individual proteins will be damaged and become resistant. Increased resistance of sets critical proteins than leads to thermotolerance expressed at the survival level. These sets could either be distributed throughout the cell or pertain to proteins within one cellular subfraction. The implications of such a set-specific resistance is that there is no single site of "inactivation" solely responsible for hyperthermic cell killing. It is to be expected that a threshold of damage exists in cells after exposure to heat; multiple sites need to be damaged before this threshold is exceeded and the cell dies. The current study pertains to the idea of such sites being distributed over at least 2 different cellular subfractions. Heat causes protein damage in both the nuclear and membrane fraction and the accumulation of this protein damage may lead to thermal killing. Thus when heat is used as an inducer of thermotolerance, resistance against the thermal protein damage is observed in both cellular fractions. The chemicals used here to induce thermotolerance may inflict damage to a different set of proteins, some of the individual proteins in that set may be identical to some of the critical proteins that are damaged by heat. It has e.g., been shown that whereas heat, ethanol and diamide at the doses used to induce thermotolerance cause significant protein aggregation in nuclei, arsenite does not [Roti Roti and Wilson 1984, Kampinga et al. 1992, data not shown]. On the other
hand, heat and arsenite causes damage to mitochondria [Yih et al. 1991], the main component of our PF showing similar transition points as the total PF [Burgman et al. in preparation], whereas ethanol does not [Yih et al. 1991, Burgman et al. unpublished observations]. Thus, the specific heat resistance of proteins seems to corresponds with the organelle (site) where these agents induced the protein damage [Kampinga et al. 1992, Burgman et al. 1993, Kampinga 1993]. If the proteins that contribute to the threshold for thermal damage are stabilized after a (chemical) triggering treatment, this will elevate the threshold level for thermal protein damage and lead to the attenuation of the heat killing effect (thermotolerance). The relationship with hsp expression and hsp reallocation with the site-specific resistance observed may be in favour of our hypothesis.

7.4.2 Site-specific resistance and heat shock proteins

Whereas all pre-treatments led to comparable TT at the clonogenic level, they did show variations in their ability to increase the cellular levels of hsp’s. Heat clearly caused the largest elevation in total cellular hsp levels whereas, for the same level of clonogenic tolerance, sodium arsenite pre-treatment is the least potent in this respect. This is especially true for hsp27 (figure 7.2a). Moreover, the various inducers of clonogenic tolerance differed in their ability to cause a redistribution of hsp70 to the particulate fraction. Interestingly, the latter could be related to the resistance of this membrane fraction (figure 7.3) and this suggests a hsp70-mediated protection against the heat-induced formation of protein damage. Such action is consistent with earlier observations that proteins of the eukaryotic hsp/hsc70 group have chaperone functions [see Hendrick and Hartl 1993, for review] and can protect proteins from aggregating after chemical or thermal denaturation. They also confirm our recent findings with hsp70 transfected rodent cells [Stege et al. 1994] in which it was found that large elevations in cellular hsp70 levels were related to an attenuation of heat-induced nuclear protein aggregation. Here, however, only marginal (not significant) attenuation of nuclear protein aggregation was seen in H-TT cells. This apparently contrast findings with rodent cells: it was found that H-TT CHO cells [Borrelli et al. 1992], H-TT rat-1 cells [Stege et al. 1994], H-TT O23 cells [Kampinga et al. 1994], and H-TT RIF-1 cells (unpublished observations) showed a reduction in the initial heat-induced aggregation of nuclear proteins. Hela S3 (but not rodent) cells apparently have such high levels constitutive hsp70 in the non-tolerant state that increments in resistance against nuclear protein aggregation are only seen after very large increases in hsp70. Indeed, recently we [see Chapter 6] observed that large accumulations of hsp70 in H-TT HeLa S3 cells could indeed lead to protection against the formation of nuclear protein aggregates in HeLa S3 cells. Such high hsp70 levels were not reached in the current study and indeed no or almost no protection against nuclear protein aggregation in TT cells was observed. Thus, only if cellular levels of hsp70 are substantially elevated above basal levels, this may lead to protection
against induction of thermal protein damage, provided that it is present (or redistributed to) the intracellular compartment when heated. In the case of the PF, hsp70 had to be redistributed to/associated with this membrane fraction in response to the tolerance triggering treatment as heating was performed on the isolated fraction. The situation for the measurement of protein aggregation in the nucleus is more complicated: here heating was done on intact cells \textit{in situ} after which these cells were fractionated to measure aggregation. Kinetics of hsp translocations to the nucleus \textit{in situ}, an effect often reported for hsp70 in particular [Ohtsuka \textit{et al.} 1986, Welch 1990] in relation to the availability of free hsp in the cell may all play a role whether or not protection against nuclear protein aggregation will be provided. In any case, no relation between the TX-100 insoluble levels of any of the hsp’s tested (both measured before or after the heat treatment) and the extent of nuclear protein aggregation could be established in the current study.

Unfortunately, up to now we were unable to measure protein denaturation and aggregation with ESR and TGA by heating isolated nuclei (like done for the PF), possibly due to interference of the spectra by DNA or RNA or due to high protein-SH content of nuclei.

The appearance of resistant proteins in the TX-100 insoluble fraction of heated HeLa S3 cells was, however, most pronounced at the level of a faster rate of recovery from protein aggregates (figure 7.4a,c). Yet, also this faster recovery from heat-induced nuclear protein aggregation as seen in H-TT, D-TT, and E-TT HeLa S3 cells (as well in all the rodent cells tested [Borrelli \textit{et al.} 1992, Stege \textit{et al.} 1994, Kampinga \textit{et al.} 1994] could not be related to the nuclear association before or after the heat treatment of cells of any of the hsp tested. Despite the fact that elevated levels of hsp27, hsc70 and hsp70 were found in the TX-100 insoluble fraction of all TT cells, no correlation with the accelerated nuclear protein disaggregation was seen. The only speculative relationship of accelerated recovery may be with the total cellular levels of hsp27. A-TT cells contained substantially less hsp27 than the other TT cells (figure 7.2a) and unlike the other TT cells showed no accelerated recovery (figure 7.4a,c). Such a relationship is supported by our recent findings that hsp27 overexpressing cells showed a more rapid disaggregation of heat-induced nuclear protein aggregates [Kampinga \textit{et al.} 1994]. Moreover, during development and decay of heat-induced thermotolerance in HeLa S3 cells, cellular hsp27 levels were found to correlate with the rates of recovery from nuclear protein aggregates [see Chapter 6]. Thus, this would suggest a role for hsp27 in repairing thermally denatured nuclear proteins from the aggregated state and that it is this protein that causes the accelerated disaggregation seen in some of the thermotolerant cells. How to reconcile such an action in terms of the currently known (chaperone) functions of hsp27 is unclear. Although data with cell free systems have suggested functions of hsp27 in attenuating (but not "repairing") heat-induced protein aggregates [Jakob \textit{et al.} 1993, Knauf \textit{et al.} 1994], such a role is not suggestive from our studies on nuclear protein
aggregation in situ [this study, Kampinga et al. 1994, see Chapter 6]. On the other hand, Lavoie et al. [1993] found that the hsp27 transfected cells were protected against heat- and actinomycin-D induced actin depolymerization. Maybe by this action, the elevated cellular hsp27 levels in the transfected system have allowed the cells to re-use other (constitutive) hsp's after heat shock in such a way that they can recover better from damage at another side in the cell. However, this remains to be elucidated.

In summary, our data suggest that elevated hsp70 levels and/or association of hsp70 with cellular subfractions can protect against induction of heat damage (protein aggregation). For the apparent more rapid recovery from (nuclear) protein damage in situ as seen in (some forms of) thermotolerant cells nor the current study, nor any study that we are aware of can provide direct insight in whether or not (a) specific hsp(s) are capable of such an action.

Unlike the situation in E. coli where DnaK (the prokaryotic hsp/hsc70 homologue) was able to rescue heat-inactivated (aggregated) RNA polymerase [Skowyra et al. 1990], the eukaryotic equivalents (alone) seem unrelated to the accelerated disaggregation of nuclear protein aggregates in situ as observed here in (some) TT cells [Stege et al. 1994, see Chapter 6, this study]. The enhancement of the ability of DnaK to reactivate RNA polymerase by DnaJ/GrpE [Liberek et al. 1991a] may suggest that in eukaryotes such combined actions also might be necessary to yield the enhanced disaggregation rate as seen in the H-TT, D-TT, and E-TT cells. Recently, the mammalian DnaJ equivalent has been identified and was shown to co-localize with hsp/hsc70 [Ohtsuka et al. 1990, Hattori et al. 1993]. It can speculated that the enhanced expression and re-allocation of this hsp40 may be the rate limiting step in protein disaggregation. On the other hand, our current and previous observation [Kampinga et al. 1994, see Chapter 6] suggest that elevated hsp27 in thermotolerant cells may (also) play a role in the observed faster recovery of thermotolerant cells from heat-induced nuclear protein aggregates.

Finally, our data suggest that heat-induced loss of clonogenic ability may be the result of accumulative heat damage in the cell. A certain threshold for damage may exist and only if this is exceeded the cell dies. The sets of proteins contributing to this threshold could be distributed throughout the cells or be within one cellular subfraction. The stabilization of only one of the sets of proteins that contribute to this threshold is then already sufficient to render cells thermotolerant at the clonogenic level.

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