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INDUCTION OF THERMOTOLERANCE IN T CELLS PROTECTS NUCLEAR DNA TOPOISOMERASE I FROM HEAT STRESS

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In this study, we have demonstrated that topoisomerase I DNA relaxing activity i protected against a severe heat shock in T cells made thermotolerant by a prior modes heat treatment. However, following a severe heat-shock challenge and incubation a 37°C, topoisomerase activity in the control population eventually returned to level similar to those detected in thermotolerant cells. This recovery of topoisomeras activity appears to result from the renaturation of heat-inactivated enzyme rather thar from synthesis of new protein because the rate of recovery of catalytic activity was no inhibited by the presence of the protein synthesis inhibitor, cycloheximide.

Elevated temperatures are known to induce a transient resistance to subsequen heat exposure in cells from a variety of organisms. This phenomenon, termed acquirec thermotolerance, is associated with the rapid and preferential synthesis of a small set o highly conserved proteins, called hsps (reviewed in 1). Hsps are induced by a numbe of apparently unrelated environmental stresses such as exposure to heavy metals alcohol, and oxidative stress, and have, therefore, also been referred to as stres proteins. The diverse group of environmental insults that can induce hsp synthesi suggest that a common induction signal, such as protein denaturation or alterec folding of newly made proteins, is responsible for hsp gene activation. Numerou studies have implicated members of the hsp70 family in protecting cells from therma damage and/or enhanced recovery from thermal stress (reviewed in 2). In addition tc protecting cells from environmental insults, members of the hsp70 family have alsc been shown to provide important functions in nonstressed cells such a depolymerization of clathrin from coated vesicles (3), and facilitation of transpor across endoplasmic reticulum and mitochondrial membranes (4,5).

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Abbreviations:
CHX, cycloheximide; con A, concanavalin A; hsps, heat shock proteins; hsc70, 70 k cognate or constitutively produced member of the hsp70 family; PEG, polyethylen glycol; ^{3}H-TdR, ^{3}H-thymidine.

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Previous studies from one of our laboratories (6) demonstrated that T cells exposed to a modest heat shock (induction temperature, 42.5°C for 60 min) were capable of resuming DNA synthesis (T cell proliferation) at an accelerated rate relative to control cells (induction temperature, 37°C for 60 min) following a subsequent severe heat-shock challenge (44-45°C for 30 min). The development of thermotolerance in T cells was associated with the induction of hsp synthesis particularly hsp90, hsc70 and the highly inducible hsp70. The close temporal association between induction of hsp synthesis and protection of T cell proliferation against heat stress suggests that hsps may be involved in protecting the cell's DNA synthetic machinery. How this might occur remains to be clarified. We have speculated that hsps may protect a critical enzyme(s) required for DNA synthesis from stress-induced damage. In this regard, DNA topoisomerases have been implicated as important enzymes in both DNA replication and transcription. Replication and transcription are associated with conformational and topological changes in DNA and topoisomerases have evolved to accommodate these changes. Two types of topoisomerases have been identified; one that produces transient protein-bridged single-strand breaks in DNA (type I topoisomerase) and another that produces double-strand breaks (type II topoisomerase, reviewed in 7). Hsps may interact with these enzymes during hyperthermic stress which may minimize thermal damage and/or accelerate restoration of their native conformation and enzymatic activity. We have, therefore, begun a study to assess whether protection of DNA synthesis against thermal injury in thermotolerant cells is associated with protection of topoisomerase I activity.

MATERIALS AND METHODS

Growth and induction of thermotolerance in T cells: Purified T cell lymphoblasts were prepared by Con A stimulation of purified splenic T cells following our previously reported procedure (6). In this study Con A activated T lymphoblasts will be referred to a T cells. After 48 -72 hrs of stimulation, T cells were exposed for one hour to a control (37°C) or modest (42.5°C) heat-shock temperature which we had previously demonstrated induces a transient thermotolerant phenotype (8). Following a recovery period of approximately 12 hrs at 37°C, both populations were given a heat-shock challenge at 44°C for 30 min. Cells were collected and either aliquoted for immediate extract preparation or evaluated for their ability to resume DNA synthesis. In some experiments CHX (50 uM, final concentration) was included in the media during the heat-shock challenge and recovery period.

Preparation of extracts: T cells ($5\text{-}30\times10^6$) were washed once with one ml PBS and lysed with high salt (300 ul 5xPBS). DNA and cell debris were precipitated with the addition of 150 ul 18% PEG containing 1.0 M sodium chloride and removed by a 10 min microfugation. The 300-400 ul of supernatant was recovered and assayed for topoisomerase activity.

Type I Topoisomerase assay: Type I topoisomerase assays were performed with supercoiled pUC19 plasmid DNA as substrate essentially as described in Lazarus et al. (9). Agarose gel electrophoresis was used to separate DNA of differing superhelical densities. One unit of topoisomerase relaxing activity removes 50% of the supercoils from the substrate in 30 min at 30°C. Data are presented as % of control following our previously reported calculation (6).

T cell proliferation assay: DNA synthesis was evaluated by incubating T cells in the presence of Con A (1.0 ug/ml) and ^{3}H-TdR (1.0 uCi/well) following our previously reported procedure (6). Following an overnight incubation (18-20 hours), cells were

harvested and the extent of DNA synthesis determined by liquid scintillatic spectrometry.

RESULTS

Fig. 1 shows the results of a typical assay for type I topoisomerase. Groups A, I and C are triplicate samples of serial dilutions (A, neat; B, 1/3; C, 1/9) of a cellul extract prepared from T cells grown at 37°C. The undiluted sample (group A) show the greatest amount of topoisomerase activity as evidenced by the nearly complet conversion of supercoiled form I DNA into more relaxed forms (I_r). Groups B and exhibit decreased amounts of topoisomerase-generated I_r DNA and increased amount of supercoiled DNA as the extract is diluted. Groups D, E, and F are triplicates c equivalent serial dilutions of T cells given a heat shock (44°C) for 30 minutes. There i a reduction in topoisomerase activity from the T cells exposed to 44°C as manifested b the reduced conversion of highly supercoiled DNA to more relaxed forms in groups D E, and F relative to those comparable samples in groups A-C. Although there is som cell death associated with the hyperthermic exposure, the results have been normalize to equivalent numbers of viable cells.

As noted above, thermotolerant T cells are able to resume DNA replication at a accelerated rate relative to control cells following a severe heat-shock challenge. It wa therefore of interest to determine whether this ability to resume DNA synthesi following thermal injury is associated with protection of type I topoisomerase. In orde to test this possibility, thermotolerant and control T cells were subjected to th indicated heat-shock challenge temperatures and then immediately assayed for residua topoisomerase activity. Aliquots of cells were also removed and tested for their abilit to reinitiate Con A-induced proliferation (i.e., resumption of DNA replication). It car be seen from the results presented in Figure 2 (left panel) that prior modest heat shock protected cellular DNA synthesis from thermal injury. We also observed marked protection of topoisomerase I activity in thermotolerant cells. For example, following a 45°C heat shock, approximately 6X more enzyme activity was detected in th thermotolerant cells versus control T cells (Figure 2, right panel). These data suggest

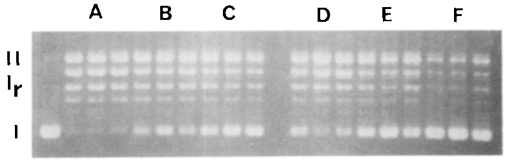

Figure 1. Gel electrophoretic separation of the products of topoisomerase-catalyzed relaxation of plasmid DNA. The first lane is control, untreated pUC19 DNA. The subsequent groups of lanes labeled A, B, and C are triplicate samples treated with neat, 1/3, and 1/9 diluted extract, respectively, from cells grown at 37°C. Groups labeled D, E, and F are triplicate samples treated with neat, 1/3, and 1/9 diluted extract from cells given a 44°C heat shock. DNA species labeled I, II, and I_r are highly supercoiled, nicked, and enzymatically relaxed circular DNA, respectively.

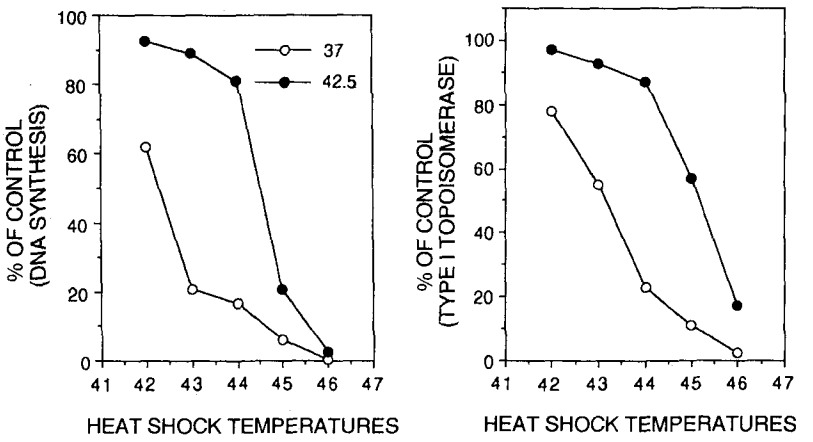

Figure 2. Relationship between resumption of DNA synthesis and type I topoisomerase activity following heat stress. T lymphoblasts were subjected to an induction temperature of either 37°C (open circles) or 42.5°C (closed circles), rested and then incubated for 30 minutes at the indicated heat shock temperatures. Immediately following heat shock, an aliquot of cells was removed to determine their ability to resume DNA synthesis (left panel). The remaining cells were used to prepare extracts for type I topoisomerase activity determinations (right panel).

hat the ability of thermotolerant cells to replicate DNA following heat stress may lepend, at least in part, on protection of this critical enzyme.

Because control cells do not recover their ability to synthesize DNA following ieat stress even after 24 hours of incubation at 37°C, we asked whether this loss was he result of continued inactivation of topoisomerase I. This hypothesis was tested by nonitoring topoisomerase activity at various time points following a heat-shock challenge. The results, presented in Fig. 3, indicate that immediately after the 44°C

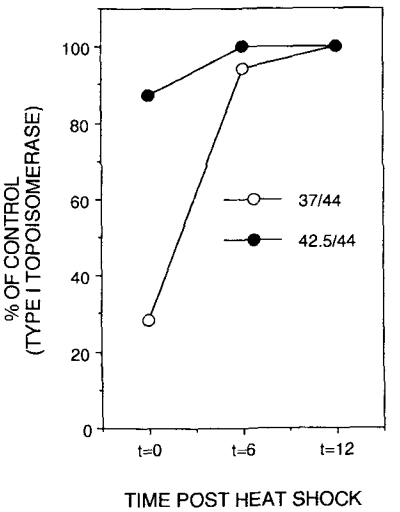

Figure 3. Effect of heat stress on type I topoisomerase activity and kinetics of recovery. T cells were incubated either at 37°C or 42.5°C and then rested for 10 hours at 37°C. Following this rest period, cells were given a 30 minute severe heat-shock challenge (44°C, 30 minutes) and either tested immediately (t=0) or at the times indicated for topoisomerase activity.

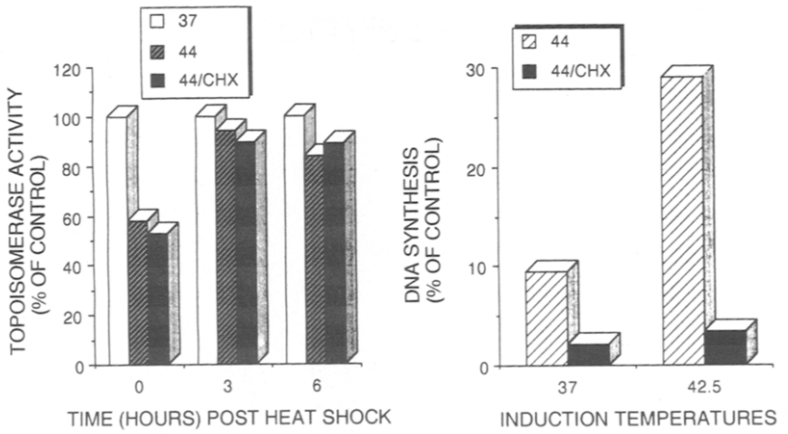

Figure 4. Failure of CHX to prevent reactivation of topoisomerase activity following heat stress. (left panel) Control cells were either incubated at 37°C or subjected to a severe heat shock (44°C, 30 minutes) in the absence or presence of CHX (50 μM/ml). Following heat shock, cells were immediately tested for either type I topoisomerase activity (T=0) or rested at 37°C for the indicated times and then assayed for topoisomerase activity. CHX remained in the culture media throughout the rest period. (right panel) An aliquot of control cells was removed immediately following heat shock in order to monitor DNA synthesis in the absence or presence of CHX . A thermotolerant population generated as described in the legend of Figure 3 was treated in a similar fashion and is included for comparison.

heat-shock challenge there was almost a four-fold higher level of topoisomerase I in th thermotolerant cells relative to the thermosensitive cells (T=0 time point) Interestingly, when heat-shocked cells were allowed to recover at 37°C, the levels c topoisomerase I in both populations of cells increased. The change in activity wa: gradual in the thermotolerant cells but most dramatic for the control cells where ther was a four-fold increase in activity within 6 hours. By 12 hrs, both the thermotoleran and control cells had equivalent levels of topoisomerase I.

In order to address whether the reappearance of topoisomerase activity ir control cells reflected translation of new topoisomerase or the stabilization o renaturation of preexisting enzyme, we performed an analogous experiment in the presence or absence of the protein synthesis inhibitor, CHX. Results shown in Fig. 4 indicate that at t = 0, there was slightly more than a 50 % reduction in topoisomerase activity in the control population. However, within 3 hours post heat shock, contro cells had recovered approximately 95% of their initial activity. This recovery occurrec despite the presence of CHX which should have inhibited production of newly translated topoisomerase. These results suggest that the recovery of topoisomerase activity is independent of *de novo* protein synthesis and most probably reflects the renaturation of heat-inactivated topoisomerase.

Results in Fig. 4 (right panel) demonstrate that these same control cells (induction temperature, 37°C) that rapidly recovered their type I topoisomerase activity, failed to resume DNA synthesis following a severe heat-shock challenge (44° even after a subsequent long-term culture (> 20 hours) at 37°C. There was even a more drastic reduction of DNA synthesis (to about 3% of control levels) in the presence

of CHX. On the other hand, the thermotolerant cells (induction temperature, 42.5 °C) exhibited a 3-fold greater capacity than the control cells for DNA synthesis in the absence of CHX. However, DNA synthesis in these thermotolerant cells was still dramatically reduced in the presence of CHX (to about 4% of control levels). Thus, these data indicate that recovery of type I topoisomerase activity is not sufficient for resumption of DNA synthesis. Reinitiation of DNA replication following a severe heat shock also requires newly synthesized protein factors that are translated on cytoplasmic ribosomes.

DISCUSSION

These initial studies indicate that (1) cells made thermotolerant by a prior modest heat shock retain almost four times the level of topoisomerase I activity as control cells immediately following a severe heat shock, (2) topoisomerase levels recover with time so that by 12 hrs after heat shock the control and thermotolerant cells exhibit the same amount of activity, (3) recovery of topoisomerase I activity appears to be independent of new protein synthesis and, therefore, probably represents the renaturation of existing enzyme, and 4) following a severe heat-shock challenge, cellular DNA replication remains impaired in control cells but recovers in thermotolerant cells and this recovery is dependent upon concurrent protein synthesis.

It should be noted that control cells rapidly recovered their type I topoisomerase activity following a severe heat-shock challenge but were still unable to resume DNA replication. This suggests that recovery from thermal injury is multifactorial. However, there are several reasons why we believe that protection and/or recovery of topoisomerase activity is an obligatory component of that recovery phase. First, studies by other investigators have demonstrated that nucleoli are very heat-sensitive (10). Second, survival of thermotolerant cells following heat stress depends upon the rapid recovery of nucleolar morphology, a process accelerated by the accumulation of hsc70 within the nucleolus (11). Third, rapid recovery of rRNA synthesis (but not mRNA or protein translation) appears to be required for expression of the thermotolerant phenotype (12). Fourth, and most importantly, eukaryotic type I topoisomerase is enriched in the nucleolus and catalytically active on ribosomal DNA (13,14). Synthesis of the precursor to 45S rRNA is dramatically reduced when HeLa cells are treated with camptothecin, suggesting an important role of topoisomerase I in rRNA transcription (15). Such a role is supported by genetic studies with yeast *top1 top2* ts double mutants which showed that, at the nonpermissive temperature, rRNA synthesis was drastically inhibited whereas tRNA and mRNA synthesis was not (16).Our own studies have recently demonstrated that hsc70 associates with type I topoisomerase *in vivo* during heat stress (unpublished observation). All these studies are consistent with the view that protection of nucleolar function and hence topoisomerase catalytic activity is crucial for the expression of thermotolerance. The fact that the cell commits a large

fraction of its constituetively produced hsp70 to this organelle and type topoisomerase also attests to the importance of protecting this enzyme from heat stres

Based on the studies presented in this report, we speculate that during hea stress hsc70 migrates into the nucleolus where it physically associates wit topoisomerase. We further suggest that this association protects the enzyme fro further damage and may be responsible for reactivation of its catalytic activity althoug how this is would be achieved remains to be clarified. As noted previously, we hav obtained direct evidence that hsc70 associates with type I topoisomerase during he stress (unpublished observation). In addition, preliminary studies *in vitro* with purifie substrates indicate that hsc70 can minimize thermal injury as well as accelerat reactivation of heat-denatured type I topoisomerase activity (unpublishe observations). These *in vitro* results mirror very closely the events that we hav described *in vivo* following heat stress and suggest that hsc70 plays a critical role i maintaining the catalytic activity of type I topoisomerase. Our preliminary studies als identify, for the first time, type I topoisomerase as a nucleolar protein targeted by hsc7 for protection against heat stress. This may explain, at least in part, the massive influ of hsc70 into the nucleolus during heat shock.

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