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Thermotolerance Inhibits Various Stress-induced Apoptosis in NIH3T3 Cells

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When NIH3T3 cells were exposed to mild heat and recovered at 37°C for various time intervals, they were thermotolerant and resistant to subsequent stresses including heat, oxidative stresses, and antitumor drug methotrexate which are apoptotic inducers. The induction kinetics of apoptosis by stresses were determined by DNA fragmentation and protein synthesis using [^{35}S]methionine pulse labeling. We investigated the hypothesis that thermotolerant cells were resistant to apoptotic cell death compared to control cells when both cells were exposed to various stresses inducing apoptosis. The cellular changes in thermotolerant cells were examined to determine which components are involved in this resistance. At first, the degree of resistance correlates with the extent of heat shock protein synthesis which were varied depending on the heating times at 45°C and recovery times at 37°C after heat shock. Secondly, membrane permeability change was observed in thermotolerant cells. When cells prelabeled with [^{3}H]thymidine were exposed to various amounts of heat and recovered at 37°C for 1/2 to 24 h, the permeability of cytosolic [^{3}H]thymidine in thermotolerant cells was 4 fold higher than that in control cells. Thirdly, the protein synthesis rates in thermotolerant and control cells were measured after exposing the cells to the same extent of stress. It turned out that thermotolerant cells were less damaged to same amount of stress than control cells, although the recovery rates are very similar to each other. These results demonstrate that an increase of heat shock proteins and membrane changes in thermotolerant cells may protect the cells from the stresses and increase the resistance to apoptotic cell death, even though the exact mechanism should be further studied.

Key words : Heat shock response, Heat shock protein, Oxidative stresses, Methotrexate, Thermotolerance, Apoptosis

INTRODUCTION

When cells are exposed to various environmental stresses including heat, oxidative stresses, heavy metal etc., they obtain the ability to resist a subsequent lethal stress. This transient heat-induced state of thermoresistance is known as thermotolerance (Hahn and Li, 1990). Although the biochemical mechanism for the thermotolerance state is unknown, the synthesis of a number of stress proteins including heat shock proteins (hsps) is involved. The enhanced synthesis of hsps has been shown to exert a protective effect on cellular survival (Lindquist and Craig, 1988). We examined the possibility that denaturation of intracellular proteins by the stresses may trigger the induction of hsp synthesis and thermotolerance. The results support the hypothesis that the production of abnormal proteins by various stresses induces the stress responses as well as tolerance (Lee and Hahn, 1988). Heat shocked or thermotolerant cells showed the resistance to environmental stress and to cell death in general.

Apoptosis is a programmed cell death distinguished from necrosis, an important process in cell elimination during the various internal (e.g. hormones, growth factor withdrawal, etc.) (Stellar, 1995; Raff *et al.*, 1993) and external stresses (irradiation, heat shock, ischemia, oxidative stress, anticancer drugs) (Takano *et al.*, 1991 and 1994; Barry *et al.*, 1990; Kaufman *et al.*, 1989). Morphologically this process is characterized by cell shrinkage, chromatin condensation and DNA fragmentation of varying degrees (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). The homeostatic control of the cell number is determined by a balance between cell proliferation and cell death. Cell proliferation is a highly regulated process with numerous checks and balances. Apoptotic cell death is a common cell death pathway that is conserved from worms to human. In principle, *BCL2* and the ced-3/interleukin-1β-converting enzyme (ICE) gene families have been identified as the genes

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involved in central cell death control. The gene *BCL2* first discovered as an oncogene, was found to have no ability to promote cell cycle progression or cell proliferation. Instead, overexpression of *BCL2* specifically prevents cells from initiating apoptosis in response to a number of stimuli (Jacobson *et al.*, 1994). Recently, it has been demonstrated that *BCL2* is only one member of a family of genes that can control the apoptotic threshold of cells (Thompson, 1995). Evidences that implicates cysteine proteases of ICE family in causing the onset of apoptosis have been accumulated. Overexpression of either *ced*-3 or ICE in mammalian cells causes apoptosis (Wang *et al.*, 1994; Kumar *et al.*, 1994). Recently it was demonstrated that activation of serine protease in addition to ICE was involved in apoptosis (Kwo *et al.*, 1995; Helgason et al., 1995). Several observations suggest that the specific inhibitor of ICE gene family may be developed for the manipulation of apoptosis. Recent understanding of apoptosis suggests that alterations in cell survival contribute to the pathogenesis of a number of human disease, including cancer, viral infections, autoimmune diseases, and AIDS (acquired immunodeficiency syndrome). Treatment designed to specifically alter the apoptosis could provide the clues for the therapy of these disease (Thompson, 1995; Stellar, 1995).

Apoptosis induced by severe stresses including hyperthermia in human T lymphocyte cell line (Mosser and Martin, 1992) or growth factor withdrawal (Mailhos *et al.*, 1993) appears to be inhibited in heat shocked cells. Many groups have performed the studies on the relationship between thermotolerance and cell survival. However, very few have defined cell survival as resistance to apoptosis (Gabai *et al.*, 1995; Samali and Cotter, 1996). Mailhos *et al.* (1994) demonstrated hsp 90 and hsp 70 protect neuronal cells from the thermal stress but not from the programmed cell death. The contradictory results may be caused from the amount of stresses and types of cell lines tested. In this study, we examined the kinetics of NIH3T3 cell survival and apoptosis induction by stresses (heat, diamide or methotrexate), the relationships among thermotolerance, prevention of apoptosis and the biochemical changes of thermotolerant cells.

MATERIALS AND METHODS

Cell lines and culture conditions

NIH-swiss mouse fibroblasts (NIH3T3) were cultured routinely at 37°C in Dulbecco's modified eagles medium (DMEM) (Gibco Ltd., Paisley Scotland, UK) with Hanks' salts supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin solution. The cells were maintained in a humidified incubator with a mixture of 95% air and 5% CO_2 and routinely checked for mycoplasma. Exponentially growing cultures containing 1×10^6 cells in 35-mm dishes were used for all experiments.

Treatment of cells with heating, diamide and methotrexate

Monolayers of cells on plastic petri dishes were heated at 45°C for various times in water baths whose temperature was controlled within ±0.1°C. The cells were reincubated at 37°C for 0-24 h for the induction of thermotolerance. Control and thermotolerant cells were then challenged with various stresses; heating at 45°C for various time, diamide (0.05~1.0 mM), or methotrexate (1~100 μM). Monolayers of cells were treated with diamide (0.05~1.0 mM) in PBS at 37°C for 30 min, rinsed and reincubated in culture medium at 37°C for various time for recovery, or with methotrexate (1~100 uM) in full media for 1-96 h to examine cell survival and DNA fragmentation induced by apoptosis. Methotrexate solutions were prepared by diluting the stock solution in DMSO to a given final concentration containing 0.1% DMSO in full media.

Cell viability and morphology

The survival assay was carried out by modification of the Sulforhodamine B (SRB) assay (Skehan *et al.*, 1990). Briefly, control or stress treated cells were trypsinized and seeded in flat-bottom 96-well plate at 200, 20 and 2K cells/well. In methotrexate treatment, seeded control were treated for various lengths of time with indicated concentrations of methotrexate in full media. Cells were incubated at 37°C for 72 h after each treatment, added with 50 μL of 50% trichloroacetic acid, left on ice for 1 h, washed with distilled water 5 times and air dried overnight. SRB solution (200 μL, final SRB concentration was 0.4% in 1% acetic acid) was added in each well, incubated at room temperature for 30 min and washed with 1% acetic acid 4 times. After air drying the plates, 200 μL of 10 mM Tris base (pH 10.5) was added in each well, solubilized the dyes by shaking. The plates were analyzed on an ELISA reader at 490 nm.

DNA extraction and electrophoresis

DNA was isolated as described previously (Baek *et al.*, 1996). Briefly, 3×10^6 cells were harvested after treatment and incubated in 0.5 mL lysis buffer (10 mM Tris, pH 7.8, 1 mM EDTA, 10 mM NaCl, 1% SDS, 0.1 mg/mL proteinase K) overnight at 37°C. After extraction of lysate with equilibrated phenol, the solution was incubated with RNase (20 μg) for 45 min at 37°C, and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and then with chloroform/isoamyl alcohol (24:1 v/v) respectively. Ethanol preci-

pitation was performed by adding 1 mL of absolute ethanol and 50 μL of 3 M sodium acetate, standing at -70°C overnight, collecting the pellet and washing with 70% ethanol by centrifugation at 22,000×g for 60 min. After the samples were dissolved in TE buffer (10 mM Tris Cl, 0.1 mM EDTA, pH 8.0) and heated at 65°C for 10 min, 10 μL aliquot was mixed with 2 μL of loading buffer consisting of 50% (w/v) glycerol and 0.02% (w/v) bromophenol blue and subjected to 1.2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After electrophoresis, the gel was soaked in TAE containing 2 μg/mL ethidium bromide and the DNA was visualized on UV light.

Pulse labeling of proteins with [^{35}S]methionine and SDS gel electrophoresis

Cells after indicated treatments were labeled with 1 μCi [^{35}S]methionine (Amersham, specific activity >1000 Ci/mmol) in 1 mL methionine free DMEM supplemented with 10% fetal bovine serum for 1 h at 37°C. After labeling, cells were washed with PBS three times and transferred to Laemmli gel sample buffer. Cell extracts were heated at 95°C for 10~20 min. Equal amounts of proteins were loaded directly onto the SDS-PAGE, and electrophoresed as described by Laemmli (1970) for reducing gel. Polyacrylamide gels were dried and exposed directly to Kodak XAR-5 film, the autoradiographs of gels were quantified by phosphoimager or BAS system.

Determination of [^{3}H]thymidine permeability

Control and thermotolerant cells were labeled by incubating with DMEM media containing [^{3}H]thymidine (1 μCi/mL) for 4 h at 37°C. After labeling, cells were washed with cold DMEM media twice. [^{3}H]Thymidine labeled control and thermotolerant cells were exposed to 45°C for 0 to 60 min and recovered at 37°C for 2 to 24 h. At each time point during the incubation at 37°C after heating, radioactivity in 100 μL of supernatant after removing the cell component was counted using Backman Liquid Scintillation Counter (Model LS 6000 TA). Fraction of radioactivity in supernatant was calculated by considering the supernatant volume.

RESULTS AND DISCUSSION

NIH3T3 monolayer cells were exposed to heat at 45°C for 20 min and reincubated at 37°C for various time periods to induce thermotolerance (TT). Control and TT cells were exposed to various stresses (heat, diamide and methotrexate) and cell survivals were determined by the SRB assay: heating at 45°C for various time (Fig. 1A); treatment with various concentra-

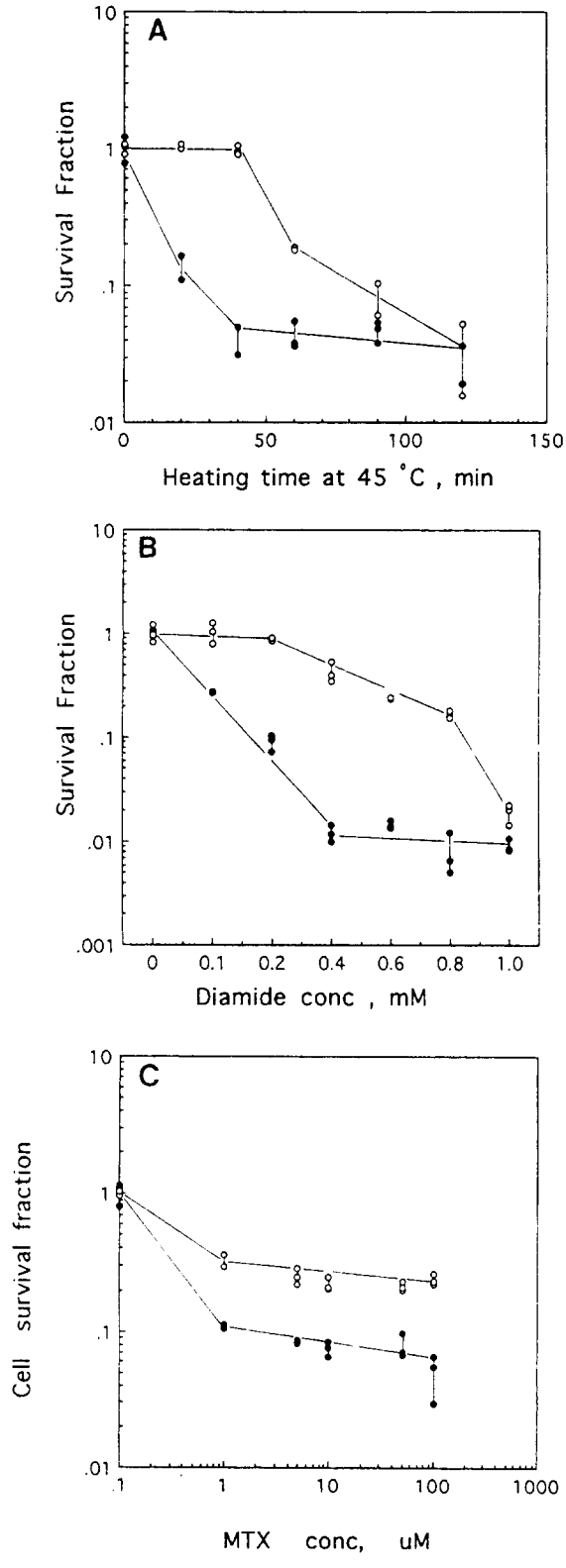

Fig. 1. Cell survival of control cells (●) and thermotolerant cells (○) when cells were exposed (A) to heat at 45°C for various time, (B) to the various concentrations of diamide in PBS for 30 min at 37°C and (C) to the various concentrations of MTX in full media for 96 h at 37°C

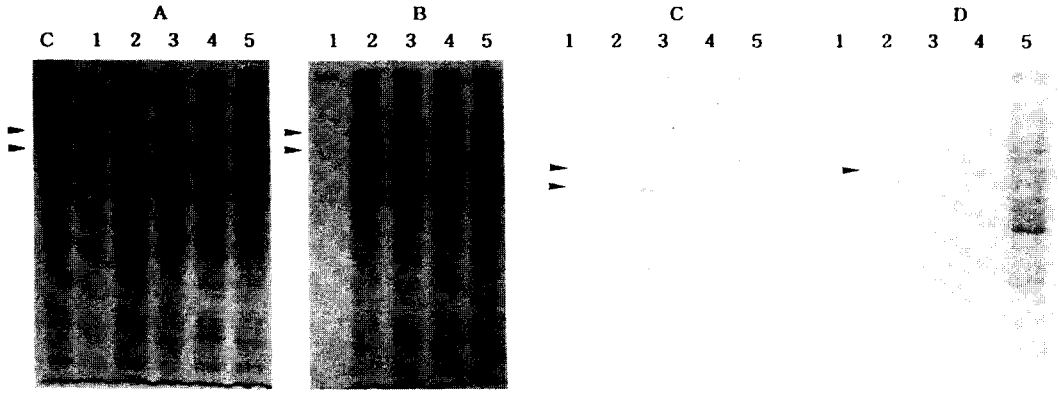

Fig. 2. Autoradiogram of [^{35}S]-methionine pulse labeled proteins from heat treated cells at 45°C. NIH3T3 cells were exposed to heat at 45°C for various time: A) 5 min, B) 10 min, C) 20 min and D) 40 min, recovered at 37°C for 1/2 h (lane 1), 3 h (lane 2), 6 h (lane 3) 11 h (lane 4) and 24 h (lane 5), and pulse labeled with [^{35}S]-methionine for 1 h. Same amount of proteins were loaded on each well. Arrows on right panel indicate the major hsp70 and hsp90. Lane C indicates untreated control cells.

tions of diamide, 0.1 to 1.0 mM in PBS, at 37°C for 30 min (Fig. 1B); exposure to various concentrations of methotrexate, 1~100 μM in full media, at 37°C for 96 h (Fig. 1C). Fig. 1 demonstrated that cells pretreated with heat (TT) clearly acquired tolerance to the subsequent diverse stress challenges; physical stress (heat), oxidative stress (diamide) and anticancer drug (methotrexate) treatment.

The rate of protein synthesis during the induction of thermotolerance was examined. Monolayers of cells were initially exposed to heat at 45°C for 5, 10, 20 and 40 min, returned to 37°C in a culture medium for various lengths of time, and then labeled with [^{35}S] methionine for 1 h at 37°C. After extraction, equal amounts of proteins were applied to each lane for electrophoresis (Fig. 2). Heating at 45°C initially inhibited protein synthesis (0.5 hr time point), but synthesis was recovered gradually during the incubation at 37°C . When the pattern of protein synthesis after heat treatment was compared with that of untreated cells, the rates of synthesis of proteins of molecular weight 89 Kda and 70 Kda were significantly enhanced at the early time points. The pattern of protein synthesis gradually returned to normal and this recovery time is depending on the extent of heating at 45°C. When cells were heated at 45°C for 5 min (Fig. 2A), protein synthesis was slightly reduced in 0.5 h after heating, the synthesis of heat shock proteins were simultaneously enhanced, and the normal protein synthesis was appeared in 6 h. Protein synthesis of cells heated at 45°C for 10 min (Fig. 2B) and 20 min (Fig. 2C) were completely stopped in 30 min after heating, then heat shock proteins were synthesized at first and the pattern of protein synthesis was returned to that of control cells afterward. Elevated synthesis of heat shock protein 70 (hsp70) during the period of thermotolerance development was observed by Western

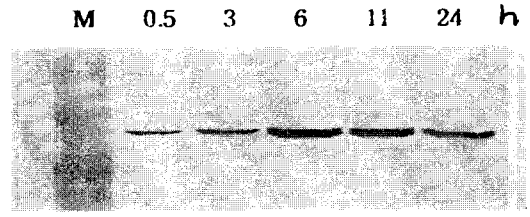

Fig. 3. Western blot analysis of induced hsp 70 in NIH3T3 cells during recovery at 37°C for indicated times after heating at 45°C for 20 min. M indicates molecular weight marker.

blot analysis using monoclonal antibody raised against hsp70 as shown in Fig. 3. When cells were given a 20 min exposure to 45°C, and returned to 37°C for 1/2 to 24 h, hsp70 was accumulated to high levels; maximum levels were attained by 6 h and maintained at this level by 24 h.

The correlations between heating at 45°C, or metothrexate treatment and apoptosis observed by DNA fragmentation pattern were shown in Fig. 4. The effect of heat shock at 45°C on the integrity of chromosomal DNA was examined. Extensive DNA fragmentation, evident as ladder of fragments, was apparent in NIH 3T3 cells which were heated at 45°C for longer than 30 min and then returned to 37°C for 24 h (Fig. 4A). It was difficult to observe the DNA fragmentation in cells heated mildly at 45°C for less than 30 min, in which the normal protein synthesis was returned in 24 h after heating. Even with strong heat shock at 45°C for longer than 30 min, DNA fragmentation was not observed immediately after the heat treatment and the extent of DNA fragmentation increased rapidly after 12 h and reached a maximal level by 24 h (data not shown). The kinetics of DNA fragmentation induced by heat stress was faster than that observed with various concentrations of anticancer drug methotrexate (Fig. 4B). DNA fragmentation induced by 1 to

50 μM methotrexate appeared after at least 96 h incubation. The extent of DNA fragmentation was proportional to the severity of the stress. When protein synthesis of cells after stress treatment was stopped for a while, the apoptotic DNA fragmentation appeared. Unlike the previous report in which the apoptotic response is prevented by the inhibitor of protein synthesis, cycloheximide (Martin *et al.*, 1990; Barry *et al.*, 1990), apoptosis in NIH3T3 cells by heat treatment was observed only when the protein synthesis was fully inhibited by stress treatment.

Many investigators have carried out studies on the correlation between thermotolerance and cell survival. However, very few have defined cell survival as resistance to apoptosis. The ability of TT cells to resist apoptosis was examined. Cells were placed at 45°C for 20 min, the condition which results in very little DNA fragmentation (Fig. 5A) and cell death, allowed to re-

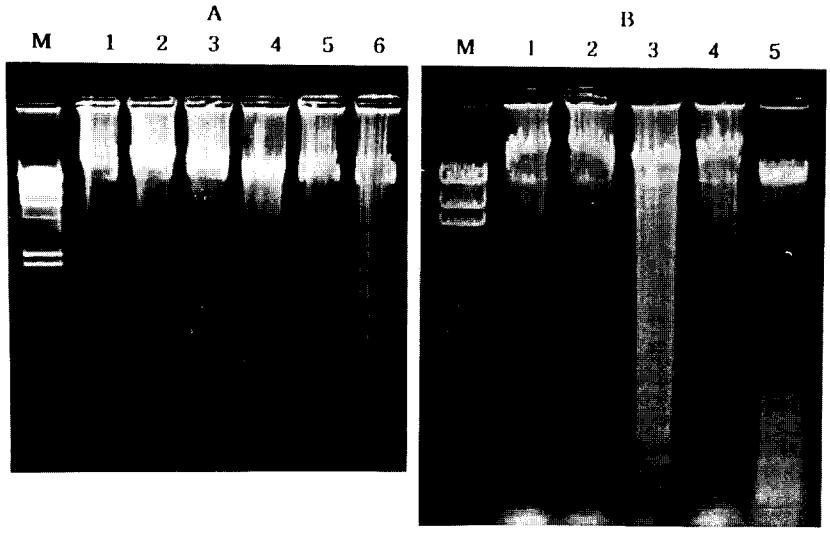

Fig. 4. DNA fragmentation pattern on agarose gel after treatment of heat (A) and MTX (B). (A) Cells were recovered at 37°C for 24 h after exposure to heat at 45°C for various time: 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 45 min (lane 5), 60 min (lane 6). (B) Cells were exposed to various concentration MTX for various time: no treatment (lane 1), 1 μM for 48 h (lane 2), 1 μM for 96 h (lane 3), 50 μM for 48 h (lane 4), 50 μM for 96 h (lane 5). M indicates the marker of λ DNA/Hind III.

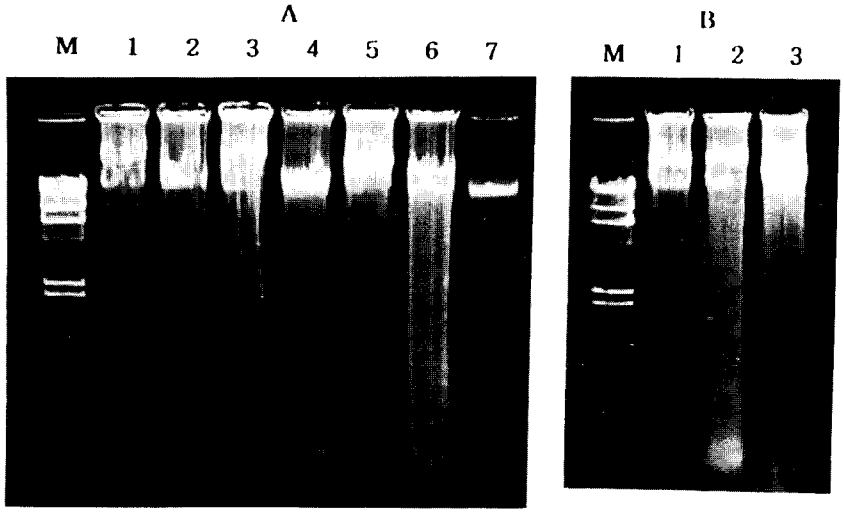

Fig. 5. DNA fragmentation pattern on agarose gel after control cells and thermotolerant cells were exposed to heat (A) and diamide (B). Thermotolerant cells were induced by recovering the cells at 37°C for 24 h after pretreatment at 45°C for 20 min. (A) Control (lane 1, 2, 4 and 6) and thermotolerant cells (lane 3, 5 and 7) were treated at 45°C for 0 min (lane 1), 30 min (lane 2 and 3), 45 min (lane 4 and 5) and 60 min (lane 6 and 7) and waited at 37°C for 24 h before DNA extraction. (B) Control cells (lane 1 and 2) and thermotolerant cells (lane 3) were treated with 0 μM diamide (lane 1) and 250 μM (lane 2 and 3) for 30 min and waited for 24 h before DNA extraction.

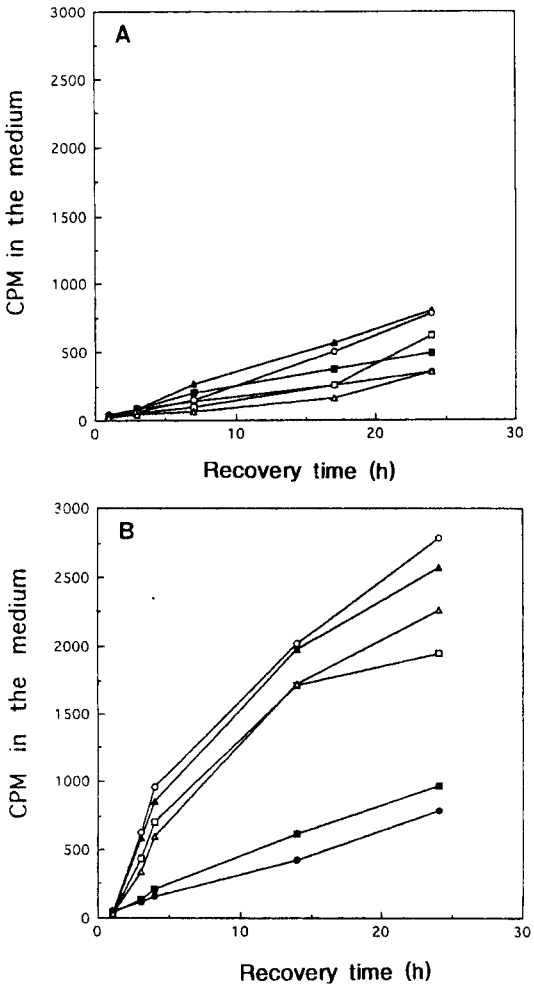

Fig. 6. Appearance of [^{3}H]-Thymidine in the media when control (A) and thermotolerant (B) cells were heated at 45°C for various time; ●, 0 min; ■, 10 min; ▲, 20 min; ○, 30 min; □, 45 min; △, 60 min, and recovered at 37°C for indicated time.

cover at 37°C for 24 h to induce TT. Control and TT cells were exposed to heat shock at 45°C for 30, 45 and 60 min, returned to 37°C for 24 hr and then collected for analysis of DNA fragmentation as shown in Fig. 5A. DNA fragmentation in control cells increased with raising the duration of hyperthermic exposure at 45°C (Fig. 5A lane 1, 2, 4 and 6) and that in TT cells exposed to the same amount of heat was significantly reduced (Fig. 5A, lane 3, 5 and 7). TT cells were also resistant to the apoptosis induced by oxidative stress with diamide treatment (Fig. 5B). This experiment indicates that thermotolerance exhibits a protective effect by reducing the extent of apoptosis induced by heat and diamide treatment in NIH3T3 cells.

It has long been questioned how TT cell could be resistant to subsequent stresses. Little is known about this mechanism except overexpression of heat shock proteins in TT cells. In this study, we have investigated the cellular and biochemical changes in TT cells. In addition to hsp 70 enhancement during induction of thermotolerance, membrane permeability change using [^{3}H]thymidine and protein synthesis rate changes after second heat treatment were examined. Control and TT cells prelabeled with [^{3}H]thymidine were washed with cold DMEM media twice, exposed to heat shock at 45°C for various time and returned to 37°C. During the reincubation at 37°C after heat shock, appearance of leaked [^{3}H]thymidine in media was measured as shown in Fig. 6. Two features are apparent: 1) [^{3}H]thymidine leakage was dramatically increased by heat shock which could damage the protein synthesis, and 2) [^{3}H]thymidine leakage in TT cells by heating (Fig. 6B) was increased about 4 fold compared with that of control cells (Fig. 6A), presumably because of membrane permeability changes. Even though the exact mechanism of membrane permeability changes is not clear, the increase of membrane permeability may play a role in resistance to various stresses of TT cells. To illustrate the mechanism of TT, control and TT cells were exposed to same amount of heat shock at 45°C for 20 min and returned to 37°C for various time, and the rates of protein synthesis were monitored by pulse labeling for 1 h with [^{35}S]methionine. Same amount of proteins were applied on each well, and the labeled actin was counted. The results indicated that TT cells were less damaged to the same extent of stresses than control cells, although the recovery rates of both cells were very similar (Fig. 7).

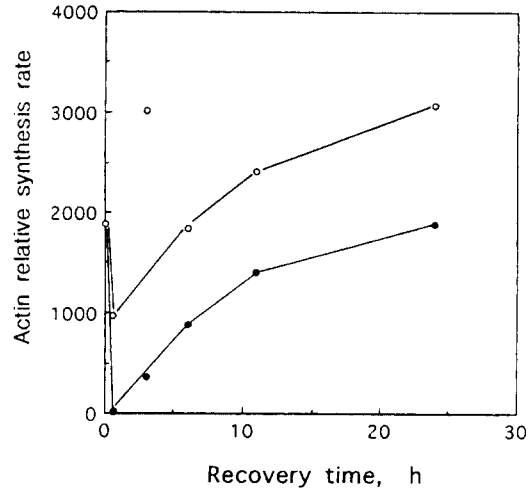

Fig. 7. The rate of actin synthesis when control (●) and thermotolerant (○) cells were exposed to same amount of heat shock (45°C, 20 min), returned to 37°C for indicated time and pulse labeled with [^{35}S]-methionine. Radioactivities of actin band on SDS-PAGE gel were counted by BAS system.

In conclusion, our data show that apoptosis has been induced by various stresses (heat shock, oxidative reagent diamide exposure, and anticancer drug metho-

trexate) which can completely inhibit the protein synthesis. Induction of thermotolerance protects against the apoptosis caused by various stresses through excess hsps productions and membrane permeability changes. TT cells may be less damaged to the same extent of stress than control cells by protecting the cells from stress induced apoptosis with increasing the heat shock proteins and changing the membrane permeability.

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