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Reduced Protein Denaturation in Thermotolerant Cells by Elevated Levels of HSP70

Mi Young Han[1] and Young-Mee Park[2]

[1]*Immune Regul. lab., Korea Res. Inst. of Biosci., and Biotech, KIST, Taejon, 305-600*
[2]*Department of Biology, Inchon University College of Natural Sciences, Inchon, 402-749*

ABSTRACT

We describe a novel approach to evaluate quantitatively the amounts of denatured proteins in cells upon heat exposure. A thiol compound, diamide [azodicarboxylic acid bis (dimethylamide)] causes protein cross-linking with exposed sulfyhydryl residues of denatured proteins. Since denatured proteins expose normally well-hidden sulfhydryl groups, these will be preferentially cross-linked by diamide. Thus diamide acts to "trap" denatured proteins. We observed that protein aggregates (high molecular weight protein aggregates, HMA) appeared on SDS-polyacrylamide gels run under non-reducing conditions and that the amount of HMA can be quantified by scanning the gels using a gas flow counter. Heating cells followed by a fixed dose of diamide exposure resulted in HMA increases in a heat-dose dependent manner, demonstrating that the quantitation of HMA could serve as a measure of heat-denatured proteins. We compared thermotolerant and nontolerant cells and found decreased HMA in tolerant cells upon heat treatment. As an attempt to examine the kinetics of protein renaturation (or "repair"), we measured the amounts of aggregates formed by the addition of diamide at various times after heat shock. Such experiments demonstrate an equally rapid disappearance of HMA in previously unheated and in thermotolerant cells. Levels of HMA in tolerant cells increased significantly after electroporation of HSP70 specific mAbs, suggesting an involvement of HSP70 in reducing HMA levels in thermotolerant cells upon heat exposure. Immunoprecipitation studies using anti-HSP70 antibody indicated an association of HSP70 with heat-denatured proteins. Our results suggest that heat induces protein denaturation, and that elevated level of HSP70 present in thermotolerant cells protects them by reducing the level of protein denaturation rather than by facilitating the "repair" (or degradation) process.

Key Words: Heat shock proteins (HSPs), Diamide, Protein denaturation, Thermotolerance

INTRODUCTION

Exposure of cells to sublethal heat treatments induces both the heat shock response and thermotolerance. The former is characterized primarily by the preferential synthesis of a series of proteins termed heat shock proteins (HSPs), while the latter is the name given to the transient heat resistance induced by a prior, nonlethal heat (or other stress) exposure. There is good temporal correlation between the appearance and turnover of HSPs and the development and decay of thermotolerance (Landry *et al.*, 1982; Li & Werb, 1982; Subjeck *et al.*, 1982). The HSPs, particularly HSP70, are thought to provide protection against damage caused by heat or other stress-inducing agents. Recent evidence has shown that overexpression of HSP70 in stably transfected rat fibroblasts was sufficient to provide an enhanced survival against heat challenge and the level of HSP70 protein expression correlated with the de-

gree of heat resistance (Li *et al.*, 1991).

On the other hand, several experiments have shown that heat shock can induce thermotolerance in mammalian cells and in yeast under conditions where no evidence for induction of any of the HSPs could be demonstrated (Carper *et al.*, 1988; Fisher *et al.*, 1992; Hallberg 1986; Ramsay 1988). These results have led Smith and Yaffe (Smith & Yaffe, 1991) to suggest that thermotolerance and HSP induction are uncoupled. Whether the HSPs, particularly HSP70, are responsible for thermotolerance, therefore, is a subject of substantial current controversy (Hightower 1991). Varieties of cellular properties have been shown to be less heat-sensitive in thermotolerant cells than in their control counterparts (Sciandra & Subjeck, 1984; Welch & Mizzen, 1988; Yost & Linquist, 1986). However, little is known about the mechanism of this protection exhibited in such cells. For these reasons, any data on possible mechanisms of how HSPs may protect cells or cellular components may be of considerable importance. HSP70 has been shown to bind and hydrolyze ATP, and the released energy has been proposed to be used for its function(s) (Chappell *et al.*, 1987; Pelham 1986). A possible role for HSP70 in lysosomal degradation of intracellular proteins in the presence of ATP and $MgCl_2$ has been proposed (Chiang *et al.*, 1989). Interaction of HSP70 with unfolded proteins and the influence of nucleotides and temperature on such interactions has been determined in cell-free systems (Palleros *et al.*, 1991). Recently, Flynn *et al.* (Flynn *et al.*, 1991) demonstrated that HSP70 can distinguish folded from unfolded proteins and those studies provided a biophysical basis for the specificity of the recognition.

Considerable evidence suggests that protein "denaturation" is responsible for heat-induced cell death (Hahn & Li, 1990; Lepock *et al.*, 1990; Minton *et al.*, 1982; Nguyen *et al.*, 1989). In view of the known properties of HSP70, we hypothesized that thermotolerant cells should be resistant to thermal denaturation of cellular proteins and that elevated levels of HSP70 should increase protein denaturation. An additional question we examined relates to the mechanism of how HSP70 may provide this resistance; Does HSP70 prevent thermal-denaturation of cellular proteins, or alternatively does it facilitate the repair of thermally-denatured proteins?

MATERIALS AND METHODS

Cells and growth conditions

The RIF-1 cells, derived from a radiation-induced fibrosarcoma as previously described (Hahn & van Kersen, 1988; Twentyman *et al.*, 1980), were cultured in RPMI 1640 (GIBCO-BRL), supplemented with 10% (vol/vol) fetal calf serum (GIBCO-BRL), 200 mg of streptomycin sulfate per liter, and 190,000 U of penicillin G potassium per liter. The cells were maintained in an humidified 37℃ incubator in a 5% CO_2-95% air atmosphere. Exponentially growing cultures containing 1-2 × 10^6 cells in 60 mm tissue culture dishes were used for all experiments. Each experiment was repeated at least three times. Part of the experiments were also repeated other cell systems which includes the mouse fibroblastoid L929 and the chinese hamster ovary subline, HA-1.

Metabolic labeling of the cellular proteins and treatment with heat and diamide

All cellular proteins were metabolically prelabeled for 48 hours with 4 μCi of L-[^{35}S]-methionine (Amersham Corp., Arlington Heights, IL.: specific activity > 1,000 Ci/mmole) per ml. A detailed protocol for thermotolerance induction, including optimum heat doses, has previously been described (Anderson *et al.*, 1989). Briefly, to allow development of thermotolerance, monolayers of cells were heated in a temperature-controlled, circulating waterbath for 15 min at 45℃ and returned to and remained in a 37℃ incubator in an atmosphere of 5% CO_2-95% air for 12~14 hrs. Control cells were treated identically, except for the priming heat shock. For the measurement of diamide-induced HMA, cells were exposed to various concentrations of diamide at 37℃. Azodicarboxylic acid bis (dimethylamide), diamide (Sigma Chemical Co., St. Louis, MO.) was stored in a refrigerated desiccator and solutions were made in Dulbecco's PBS (GIBCO-BRL) immediately prior to use. For the measurement of heat-induced HMA, cell were exposed to elevated temperatures for increasing time intervals immediately followed by addition of a fixed dose of diamide.

One dimensional polyacrylamide gel electrophoresis

Cellular proteins were solubilized in SDS-sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), with 8% acrylamide for separating gels. The procedure was essentially identical as described by Laemmli (Laemmli 1970). Protein concentration was measured by the method of Lowry *et al.* (Lowry *et al.*, 1951). For the quantitation of HMA, SDS-PAGE was carried out under nonreducing conditions. The gels were then stained, dried, and scanned on a proportional gas flow counter (AMBIS Scanner, Automated Microbiology Systems, San Diego, CA.). The amount of protein denaturation was measured by determining % CPM contained in HMA relative to the total CPM in each lane. Autoradiograms were prepared using Kodak XAR-2 film.

Western blot analysis

Immediately after electrophoresis, the proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Bio-Rad) by electroblotting (E. C. Apparatus, St. Petersburg, Fla.). Proteins transferred to the membrane were probed with a mouse monoclonal antibody (mAb), N6 F3-5, which recognizes both the constitutive and inducible forms of HSP70. Binding of the mAb to HSP70 was detected by incubation of the blot with an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Bio-Rad). 3-hydroxy-2-naphthoic acid 2,4-dimethyanizide (Sigma)/Fast Red TK dye (Bio-Rad) were used as substrates for AP reaction.

Immunoprecipitation

Prelabeled cells were heated at 45℃ for increasing time intervals, and processed for immunoprecipitation. Briefly, protein A-mAb complex was prepared by incubating protein A-Sepharose beads (Pharmacia) with mAb against HSP70 under high salt conditions (140 mM Na_2HPO_4, pH 9.0, 2.5 M NaCl). The incubation was carried out at 4℃ in a vertical rotator. The complex was pelleted and washed twice with the incubation buffer and then twice with NP40-lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP40). Monolayers of cells were washed twice with room temperature PBS, lysed with NP40-lysis buffer supplemented with protease inhibitors and centrifuged for 10 min at 10,000×g. The cell extracts were transferred to a clean tube. Aliquots of cell extracts and prepared protein A-mAb were incubated at 4℃ in a vertical rotator. The immune-complexes were collected by centrifugation at 10,000×g for 15 sec at 4℃, resuspended in lysis buffer, and washed three times with lysis buffer. The final wash was completely removed by inserting a bent 23 gauge needle directly into the beads. The radioactivity in aliquots of each sample was measured, and the samples were further processed for SDS-PAGE by solubilizing an equal amount of radioactivity for each immune complex with SDS-sample buffer.

Electroporation of mAb against HSP70

The Cell-Porator Electroporation System (BRL, Bethesda, MD) was used to introduce mAb into cells. Exponentially growing cells were trypsinized and washed with HEPES-buffered saline (21 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose). Cells were resuspended at a density of 2×10^6 cells/ml. Two hundred fifty microliters of cells were transferred to an electroporation chamber containing mAb at a final concentration of 3 mg/ml and exposed to a single electric pulse of 750 V/cm for duration of 0.7~0.9 msec at 4℃. Cells were left in the chamber for 5 min, washed with growth media, and returned to a 37℃ incubator. To confirm the successful introduction of mAb, N6, electroporated cells were harvested, washed in PBS, and lysed in SDS-sample buffer. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The heavy and light chains of the introduced mAb were identified using AP-conjugated goat anti-mouse antibody. The successful introduction of the mAb was further confirmed by electroporating fluorescein isothiocyanate (FITC)-labelled mAb into cells. Following washing in PBS, the cells were plated into 35 mm tissue culture dishes and put back for 37℃ incubator for 4~6 hrs. Then the dishes were washed again in PBS, fixed with −20℃ absolute methanol, and kept at −20℃ for 16 hrs. ACAS (Adherent Cell Analysis and Sorting, Meridian Instrument, Okimos, MI) was used to determine FITC-labelled

N6-uptake by electroporated cells. Values for the integrated fluorescence per cell (IF/cell) were obtained by scanning mAb electroporated cells (excitation beam 488 nm, argon laser; emission filters 530 ± 15 nm band pass).

RESULTS

Measurement of protein denaturation induced by heat

In order to evaluate quantitatively the amounts of denatured proteins in cells after heating, we have developed a method using diamide [azodicarboxylic acid bis (dimethylamide)], a sulfhydryl oxidizing agent. Diamide interacts with sulfhydryl residues, randomly cross-links them, and therefore produces high molecular weight protein aggregates (Lee & Hahn, 1988). We defined high molecular weight protein aggregates (HMA) operationally as either those proteins that did not enter the gel or those that appeared between the stacking and the resolving gel on a SDS-polyacrylamide gel run under non-reducing conditions. These could readily be quantified as described in Materials and Methods. We found that the amounts of HMA increased as the concentration of diamide increased and reached a plateau at 5~10 mM, suggesting that diamide is trapping denatured proteins present in cells at the time of exposure (Fig. 1). When the cell extracts obtained from those that were exposed to diamide were subjected to a gel eletrophoresis under reducing condition, HMA completely disappeared and var-

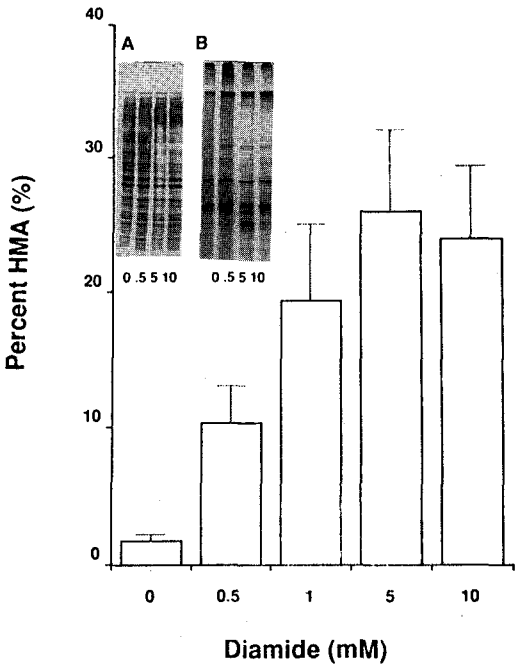

Fig. 1. Increase in HMA formation correlates with the concentration of diamide added to RIF-1 cells. All the cellular proteins were prelabeled with L-[^{35}S]-methionine before treatment as described in Materials and Methods. The insert shows total protein samples of RIF-1 cells treated with diamide run under reducing condition (A) and non-reducing condition (B). Error bars represent mean ± SD.

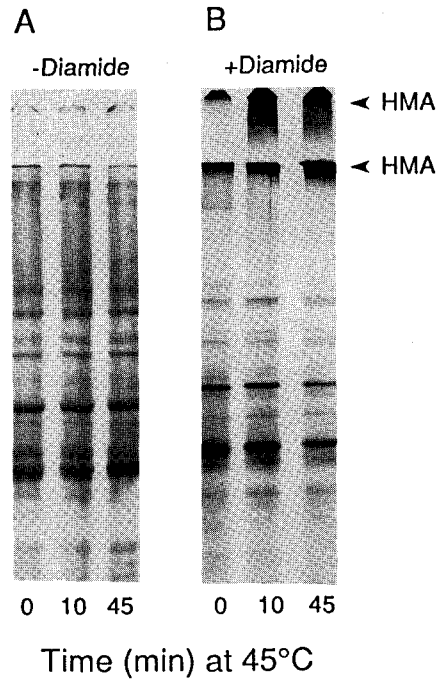

Fig. 2. Appearance of HMA after diamide exposure following heating. Prelabeled RIF-1 cells with L-[^{35}S]-methionine were heated with (A) or without (B) diamide addition (0.2 mM/30 min) and cell extracts were subjected to SDS-polyacrylamide gel electrophoresis under non-reducing condition.

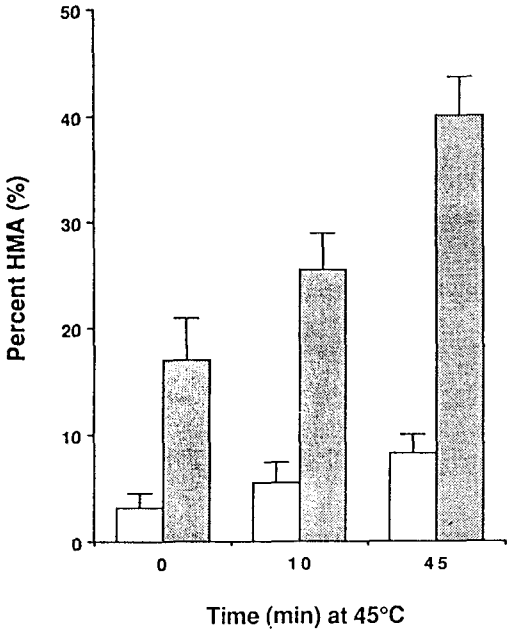

Fig. 3. Increase in HMA production in RIF-1 cells is heat dose dependent. RIF-1 cells were heated at 45℃ for 0 to 45 min and exposed to diamide (0.2 mM/30 min). Quantitation of HMA was performed by scanning gas flow counter as described in Materials and Methods (dark bars). Data obtained from samples not exposed to diamide but to PBS following heating are shown for comparison (light bars). Error bars represent mean ± SD.

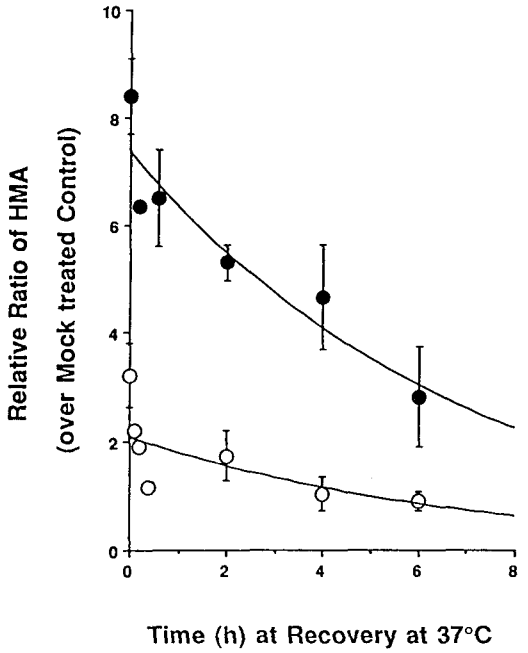

Fig. 4. A time course of HMA disappearance at 37℃ in thermotolerant cells (white circles) and in nontolerant cells (black circles). Cells were heated for 15 min at 45℃ and put back to 37℃ incubator for 0 to 6 hrs. Diamide (0.2 mM/30 min) was exposed either immediately after heating or at the end of the incubation period. Quantitation of HMA was done as described in Materials and Methods. Error bars represent mean ± SD.

ious protein bands of different molecular weight appeared instead in the resolving portion of the gel (Fig. 1, panel A of insert), suggesting that diamide is trapping denatured proteins randomly. We reasoned that heat-induced denatured proteins would expose normally sequestered hydrophobic regions of proteins including sulfhydryl groups, and thus could be more readily cross-linked by diamide. In the absence of diamide exposure, we observed little differences in the amounts of HMA in cells heated for various lengths of time (Fig. 2, panel A). When the cells were exposed to a fixed dose of diamide (0.2 mM/30 min) immediately following increasing time of heating, however, the appearance of increasing quantities of HMA was found (Fig. 2, panel B). As shown in Fig. 3, there was a correlation between the heat dose given and the amounts of HMA, which lead us to conclude that the amount of heat-denatured proteins can be mirrored by the level of HMA trapped by diamide following heating.

Thermotolerant cells are protected against HMA formation induced by heating

Since thermotolerant cells display enhanced survival upon otherwise lethal heat shock, we examined whether there is a difference in the level of HMA formation between thermotolerant and nontolerant counterparts. We compared two features: the initial levels of heat induced-HMA and the rate of HMA disappearance. The latter would presumably reflect the sum of "repair" and degradation of denatured proteins. In these experi-

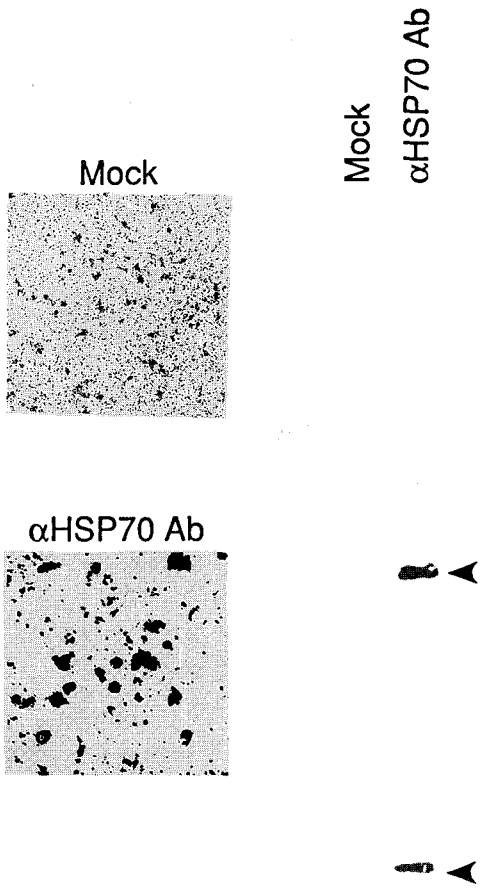

Fig. 5. Incorporation of HSP70 specific mAb in RIF-1 cells. Left panel: Introduction of FITC-conjugated HSP70 specific mAb was visualized by ACAS (aHSP70 Ab). Right panel: Western blot analysis of the HSP70 specific mAb in RIF-1 cells after electroporation.

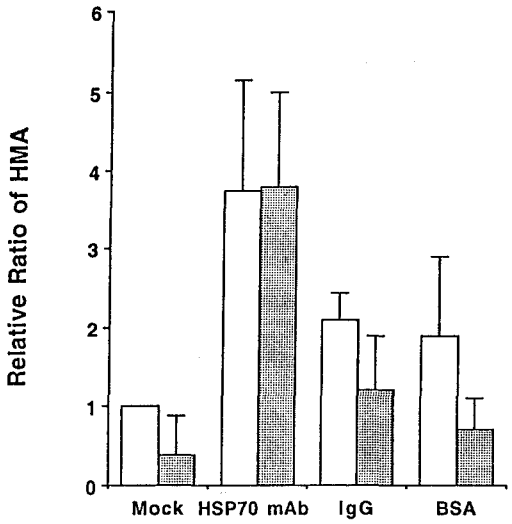

Fig. 6. The effect of electroporation of HSP70 specific mAb, isotype control IgG1, or BSA on the accumulation of HMA in RIF-1 cells heated at 45℃ for 15 min and exposed to diamide (0.2 mM/30 min). Light bars are nontolerant RIF-1 cells and dark bars are theromotolerant RIF-1 cells. Error bars represent mean ± SD.

ments, both tolerant and nontolerant cells were heated at 45℃ for 20 min. Immediately after heating, the cells were exposed to a fixed dose of diamide and processed in order to compare initial HMA induction by heat, or returned to a 37℃ for increasing time intervals in order to examine the rate of HMA disappearance. As shown in Fig. 4, the initial quantity of HMA formation was significantly lower in thermotolerant cells than in nontolerant cells. Further, during the recovery at 37℃, the amount of HMA decreased at an exponential rate in both thermotolerant and nontolerant cells. In both cells, the amount of time required for the amount of initial damage to be reduced to 1/e of the original value (the relaxation time) was 3. 5 ± 1.2 hr. These data demonstrate an equally rapid disappearance of HMA molecules in previously unheated and in thermotolerant cells. While the tolerant state reduces the amount of initial protein damage caused by heat shock, renaturation (or "repair") process does not appear to be more rapid in thermotolerant cells.

We questioned whether the observed difference in the quantity of HMA between thermotolerant and nontolerant cells was due to the difference in the uptake of diamide. To do so, we have compared the rate of depletion of a major intracellular free thiol, glutathione after diamide exposure by Fluorescence Activated Cell Sorter (FACS) (Rice *et al.*, 1986). This assay is based on the intracellular reaction of monocholorobimane with glutathione to form a highly fluorescent adduct which is trapped within cells. The rate of reduction in fluorescence was similar between the

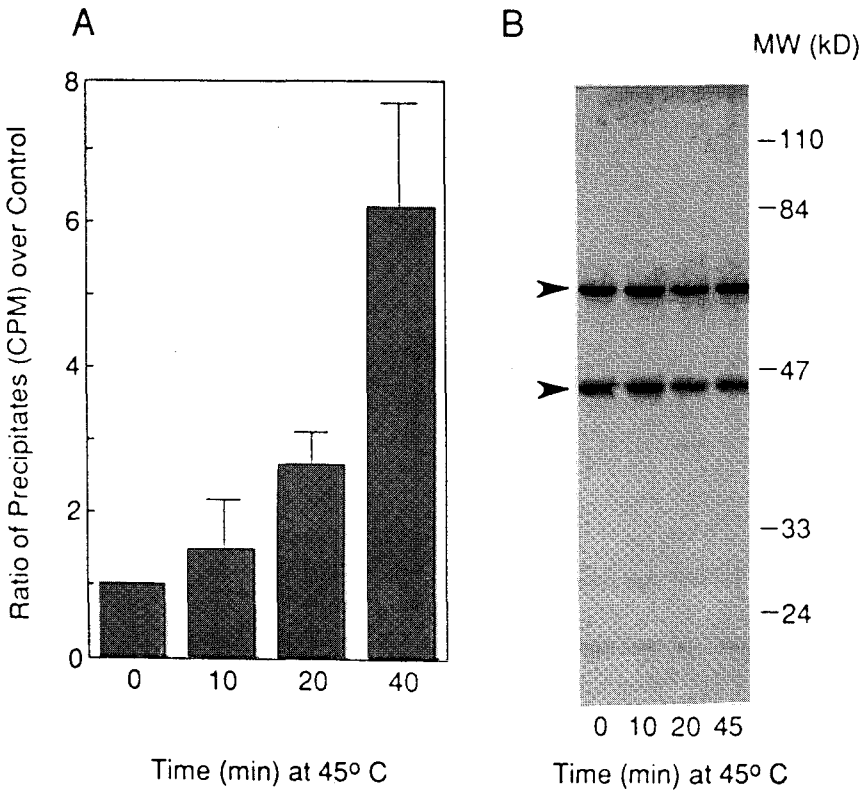

Fig. 7. Panel A: Increased amounts of HSP70 coprecipitates in metabolically labelled cells heated at 45℃ for 0 to 45 min. Error bars represent mean ± SD. Pancel B: Western blot analysis of HSP70 coprecipitates from A under reducing condition. The blot was probed mAb against HSP70.

thermotolerant and control cells, showing that the differences in the amounts of HMA did not simply reflect the differences in the uptake of diamide (data not shown).

The effect of electroporation of HSP70 specific mAb in HMA production

To examine whether the level of heat induced HMA production were modulated by the level of HSP70 present in cells during heating, we attempted to inactivate "functional" HSP70 by electroporating HSP70-specific mAb into cells. To confirm the successful introduction of mAb after electroporation, the fluorescein isothiocyanate (FITC)-conjugated HSP70-specific mAb was introduced into cells and examined by ACAS as described in Materials and Methods (Fig. 5, left panel): back ground fluorescence from cells fixed before the electroporation (Mock) is shown in comparison with the accumulated fluorescence of the cells after the electroporation of FITC-conjugated mAb (anti HSP70 Ab). We have also checked the integrity of the mAb after the electroporation. To do so, aliquots of samples of RIF-1 cells were subjected to Western blot analysis after the HSP70-specific mAb electroporation. As shown in right panel of Fig. 5, neither the heavy nor the light chain of the incorporated mAb showed detectable degradation, indicating that the electroporation procedure itself did not cause mAb destruction. Both the heavy and the light chains can be visualized. We then examined the effect of electroporation of HSP70 specific mAb in HMA production upon heat exposure. As shown in Fig. 6, in the presence of electroporated monoclonal anti HSP70 Ab, the quantity of HMA

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=국문초록=

열내성이 유도된 세포에서 HSP70 단백질 증가에 의한 단백질 변성 감소

생명공학연구소 면역학실험실, 인천대학교 자연과학대학 생물학과

한 미 영[1] · 박 영 미[2]

본 연구에서는 열충격에 의한 세포내 단백질 변성을 정량하는 방법을 소개하고 있다. Thiol compound인 diamide [azodicarboxylic acid bis (dimethylamide)]는 단백질변성시 노출된 sulfyhydryl기를 cross-link 시킨다. 정상 상태에서는 노출되지 않는 sulfyhydryl group이 변성된 단백질에서는 노출되기 때문에 diamide에 의한 cross-linking이 선택적으로 일어날 것이다. 그러므로 diamide는 변성된 단백질을 "trap"하는 작용을 할 수 있다. 본 연구진은 세포내 열충격후 고분자 단백질 응집물 (high molecular weight protein aggregate, HAA)이 나타남을 비환원 (non reducing) SDS-PAGE에서 관찰하였고 이를 gas flow counter로 scanning하여 정량하였다. 실험 결과 세포에 열충격을 가한후 diamide를 처리하면 HMA가 열충격 용량 의존적으로 증가함을 관찰하였다. 이는 HMA의 양을 측정함으로써 열충격에 의하여 변성된 단백질을 정량할 수 있음을 반증한다. 열내성이 유도된 세포와 그렇지 않은 세포를 비교하였을 때 열내성이 유도된 세포에서는 열충격에 의한 HMA의 형성이 억제됨을 관찰하였다. 열충격후 정상 온도에서 회복기를 주면서 시간대별로 diamide를 첨가하고 이때 형성된 HMA양을 측정하여, 단백질 원형복구의 역동성을 실험하였다. 그 결과, HMA는 열내성의 유도 여부와 상관없이 빠르게 없어짐을 알 수 있었다. 그러나 열내성이 유도된 세포에서 HSP70 단항체를 electroporation에 의하여 투여하였을 때 HMA가 현저히 증가하였고, 이는 열내성이 유도된 세포에서는 HSP70의 증가에 의하여 HMA생성이 억제되었음을 나타낸다. HSP70 항체를 이용하여 면역침전을 시행한 결과 변성된 세포내 단백질이 HSP70과 같이 침전됨이 관찰되었다. 이 결과는 HSP70 단백질이 변성된 단백질과 일시적으로 결합하여 정상 상태로 돌아가거나 복구될 수 있도록 도와줄 수 있음을 시사한다.