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Integrity of Intermediate Filaments Is Associated With the Development of Acquired Thermotolerance in 9L Rat Brain Tumor Cells

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Abstract Withangulatin A (WA), a newly discovered withanolide isolated from an antitumor Chinese herb, has been shown to be a vimentin intermediate filament-targeting drug by using immunofluorescence microscopy. Together with cytochalasin D and colchicine, these drugs were employed to investigate the importance of vimentin intermediate filaments, actin filaments, and microtubules in the development of acquired thermotolerance in 9L rat brain tumor cells treated at 45°C for 15 min (priming heat-shock). Acquired thermotolerance was abrogated in cells incubated with WA before the priming heat-shock but it could be detected in cells treated with WA after the priming heat-shock. In contrast, cytochalasin D and colchicine do not interfere with the development of thermotolerance at all. The intracellular localizations of vimentin and the constitutive heat-shock protein70 (HSC70) in treated cells were examined by using immunofluorescence microscopy and detergent-extractability studies. In cells treated with WA before the priming heat-shock, vimentin IFs were tightly aggregated around the nucleus and unable to return to their normal organization after a recovery under normal growing conditions. In contrast, the IF network in cells treated with WA after the priming heat-shock was able to reorganize into filamentous form after a recovery period, a behavior similar to that of the cells treated with heat-shock only. HSC70 was found to be co-localized with vimentin during these changes. It is suggested that the integrity of intermediate filaments is important for the development of thermotolerance and that HSC70 may be involved in this process by stabilizing the intermediate filaments through direct or indirect binding.

Key words: thermotolerance, heat-shock response, cytoskeletal systems, vimentin, HSC70

Cells respond to supraoptimal temperatures by reprogramming a series of metabolic processes which are collectively termed the heat-shock response [for reviews, see Lindquist, 1986; Nover, 1991]. The hallmark of a heat-shock response is the induction of heat-shock proteins (HSPs) which are thought to be able to protect cells from further damage under the stressful condition [Lindquist and Craig, 1988; Schlesinger, 1990; Welch, 1992]. When recovering from a sublethal heat-shock, cells in culture are usually able to develop a transient resistance to a subsequently lethal heat-shock challenge, a phenomenon known as acquired thermotolerance [Gerner and Schneider, 1975]. This phenomenon has been extensively studied at the level of cell survival and it falls into two distinct categories: one that requires de novo synthesis of proteins and one that does not [Laszlo, 1988; Lee and Dewey, 1988; Borrelli et al., 1993]. The protein synthesis dependent thermotolerance protects cells by some mechanisms involving newly synthesized HSPs, based upon the quantitative correlation established between enhanced synthesis of HSPs and development of acquired thermotolerance [Henle and Dethlefsen, 1978; Li and Werb, 1982; Landry et al., 1989; Liu et al., 1992]. On the other hand, thermotolerance may develop in the absence of HSP synthesis (protein synthesis independent thermotolerance), indicating that de novo synthesized HSPs are not involved in the protection mechanism [Anderson et al., 1986; Bader et al., 1992; Fisher et al., 1992]. It has been suggested that a nonlethal heat-shock may cause changes in the reac-

Abbreviations used: AFs, actin-containing microfilaments; CC, colchicine; CD, cytochalasin D; HSC70, the constitutive member of the HSP70 family; HSP, heat-shock protein; IFs, intermediate filaments; MTs, microtubules; RBT cells, rat brain tumor cells; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; WA, withangulatin A.

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tivity of constitutive expressed HSPs that confers acquired thermotolerance [Landry et al., 1991]. Furthermore, protein synthesis independent thermotolerance may also occur by a mechanism that does not involve proteins. For instance, it has been suggested that physical and compositional alterations of membrane lipid components during and subsequent to heat-shock may, at least in part, account for the cell adaptation process [Yatvin and Cramp, 1993].

Cytoskeleton consists of three major filamentous systems, intermediate filaments (IFs), actin-containing microfilaments (AFs), and microtubules (MTs), which together form an extensive and intricate protein scaffold in the cytoplasm [Bershadsky and Vasilier, 1988]. It appears to function in cell shape, cell motility, transportation of intracellular macromolecules, translation of mRNA into protein, and regulation of gene expression [Bershadsky and Vasiliev, 1988; Zambetti et al., 1991]. In heat-shocked cells, all of the three cytoskeletal systems, IFs, AFs, and MTs, have been shown to be disrupted [Glass et al., 1985; Welch and Suhan, 1985; Welch et al., 1985; van Bergen en Henegouwen et al., 1985; van Bergen en Henegouwen and Linnemans, 1987]. The perturbations of the cytoskeleton are thought to be related to or responsible for the development of thermotolerance [Welch and Mizzen, 1988]. Nevertheless, the role(s) of each individual cytoskeletal system in the development of acquired thermotolerance has not been elucidated.

Withangulatin A (WA) is a novel stress inducer isolated from the Chinese antitumor herb *Physalis angulata* [Chen et al., 1990; Lee et al., 1991]. It disrupts vimentin IFs in vivo and the alteration is accompanied by biochemical changes of vimentin molecules, including augmentation of phosphorylation, retardation of electrophoretic mobility, and decrease in detergent extractability [Lee et al., 1993]. Cytochalasin D (CD) is an AFs-disrupting drug which reversibly binds to the barbed end of AFs and thus inhibits actin polymerization [Flanagan and Lin, 1980; Casella et al., 1981]. Colchicine (CC) inhibits the polymerization of tubulin by forming molecular complexes with nonpolymerized tubulin which then bind to both ends of the MTs to decrease association rate [Margolis and Wilson, 1977; Bergen and Borisy, 1983]. In this study, the specificity of WA on the IF network was further studied and the above drugs were used to examine the role(s) of individual cytoskeletal systems in relation to the development of thermotolerance in 9L RBT cells. In addition, the possible involvement of HSC70 in this process was also investigated.

MATERIALS AND METHODS

Materials

WA was isolated from *Physalis angulata* and kindly provided by Dr. C.-M. Chen of the National Tsing Hua University [Chen et al., 1990]. It was dissolved in dimethyl sulfoxide at a concentration of 20 mM and stored in the dark at 4°C. CD and CC were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in dimethyl sulfoxide at a concentration of 1 mM and stored in the dark at −20°C. The above stocks were diluted to appropriate concentrations with culture medium before use. All cultureware was obtained from Corning (Corning, NY) or Nunc (Roskilde, Denmark). Culture medium components were purchased from GIBCO (Gaithersburg, MD). Monoclonal antibodies against cytoskeleton components and HSC70 were from Amersham (Buckinghamshire, England) and StressGen (Victoria, BC, Canada), respectively. Other supplies for immunoblotting and immunofluorescence microscopy were purchased from Amersham or BioRad (Richmond, CA). Chemicals for electrophoresis were obtained from BioRad and general chemicals were supplied by Sigma or Merck (Darmstadt, Germany).

Cell Culture

The 9L rat brain tumor (RBT) cells were a generous gift from Dr. M. L. Rosemblum, University of California at San Francisco [Weizsaecker et al., 1981]. They were maintained in Eagle's minimum essential medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μM/ml streptomycin. Cells were kept in a humidified 37°C incubator with a mixture of 5% CO_2 and 95% air. Stock cells were plated in 25 cm^2 flasks or 6-well plates at a density of 4 to 6×10^4 cells per cm^2. All experiments were performed by using exponentially growing cells at 80–90% confluency. Cell survivals were determined by colony formation technique. After treatment, cells were trypsinized, serially diluted, and counted with a hemocytometer. The plating efficiency was determined by seeding in duplicated dishes at appropriate density of cells per 60 mm dish containing 4 ml of culture

medium. The plated cells were then incubated at 37°C for 8 to 10 days. Subsequently, the samples were rinsed with phosphate-buffered saline (PBS), stained with 1.5% methylene blue in PBS, drained, and rinsed gently under running water. The colonies formed with more than 50 cells were scored. The plating efficiency of 9L RBT cells was 60 to 90%. Surviving fraction of the treated cells was referred to as the fraction of plating efficiency relative to that of untreated controls.

Heat-Shock and Drug Treatments

In the heat-shock experiments, flasks or plates were sealed with Parafilm and submerged for various durations in a water bath pre-set at 45 ± 0.1°C. The designated temperature of the medium in the heating protocol was reached within 3 min and the time required for equilibrium was included in the treatment duration. For drug treatments, stock solutions were diluted with culture medium to the specified concentrations before adding to the cells.

Acquired thermotolerance was induced in 9L RBT cells by a priming heat-shock at 45°C for 15 min, followed by a recovery period at 37°C, and then challenged with a second heat-shock at 45°C for 60 min. To study the effects of WA, CD, and CC on the development of acquired thermotolerance, drugs were added either 45 min prior to the priming heat-shock which was performed in the presence of the drugs (pre-priming drug treatment) or immediately after the priming heat-shock and the cells were incubated at 37°C in the presence of the drugs for 1 h (post-priming drug treatment). After various durations at 37°C (which were defined as the recovery periods), cells were then challenged with a second heat-shock at 45°C for 60 min and cell survivals were then determined as described above.

Indirect Immunofluorescence and Microscopy

For indirect immunofluorescence microscopy, cells were grown on chamber slides (Nunc). After treatments, the slides were briefly washed with PBS and the cells were then fixed and permeabilized with methanol at −20°C for 10 min. After a brief rinse in PBS, the fixed cells were incubated for 1 h at room temperature with mouse monoclonal antibodies against vimentin, actin, tubulin, or HSC70 (diluted 1:20, 1:50, 1:50, and 1:100, respectively). The antibodies were diluted in PBS containing 3% BSA and their specificity was pre-tested by immunoblotting techniques. Cells were then washed with PBS and incubated with fluorescein-conjugated goat-anti-mouse IgG (diluted 1:20) for 1 h at room temperature. After another rinsing with PBS, the cells were mounted in glycerol for examination. A Nikon microscope equipped with epifluorescence optics (Nikon Optiphot, Tokyo, Japan) was used for obtaining photographs of the fluorescent images.

Protein Extraction and Cell Fractionation

Cellular proteins were separated into "extractable" and "non-extractable" fractions before they were subjected to gel electrophoresis as described [Lee et al., 1992, 1993]. After treatments, cells in 6-well plates were washed once with PBS at room temperature and incubated with 0.2 ml/well of extraction buffer (1% nonident P-40 and 0.15 M NaCl in 50 mM Tris-HCl, pH 8.0) on ice for 10 min. Soluble proteins extracted under this condition were collected into microcentrifuge tubes and the samples were centrifuged at 12,000*g* for 10 min at 4°C. Aliquots of the supernatants, referred to as the extractable fraction of cellular proteins, were added to equal amounts of double-strength sample buffer (0.0625 M Tris-HCl, pH 6.8; 2% sodium dodecylsulfate, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) before electrophoresis. Cellular proteins remaining on the surface of the culture dishes were rinsed three times with ice-cold PBS and solubilized in 0.2 ml of sample buffer before electrophoresis. The samples thus collected were referred to as the non-extractable fraction of the cellular proteins and presumed to be the nuclear-cytoskeletal fraction as previously reported [Collier and Schlesinger, 1986; Lee et al., 1992].

Gel Electrophoresis and Immunoblot Analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970]. The samples in sample buffer for SDS-PAGE were heated in boiling water for 5 min and then centrifuged (Eppendorf, full speed) for 3 min before loading. They were applied to 10% SDS-polyacrylamide gels on the basis of equal amount of cell lysate. For immunoblotting, the gel was soaked in transfer buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) for 10 min after electrophoresis and the resolved proteins were then electrotransferred onto a nitrocellulose membrane (Hy-

bond-C extra, Amersham) by a semi-dry method (OWL Scientific Plastics, Inc., Cambridge, MA). The membrane was incubated for 1 h with 3% gelatin in Tween containing Tris-buffered saline (TTBS: 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS briefly. Subsequently, the membrane was incubated with monoclonal antibodies to HSC70 (diluted 1:1,000 in TTBS containing 1% gelatin) at room temperature for at least 2 h. After three washes with TTBS, immunocomplexes on the membranes were reacted with goat-anti-mouse antibody conjugated with alkaline phosphatase (diluted 1:2,000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membrane was then rinsed three times with TTBS, dried, and developed into a color immunoblot at room temperature in developing buffer (15 mg of nitro blue tetrazolium, 0.7% N,N-dimethylformamide, 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml, 1 mM $MgCl_2$, and 100 mM $NaHCO_3$, pH 9.8). Bands of interest on the immunoblots were quantitated by densitometric scanning in 2-D mode (Molecular Dynamics, San Francisco, CA).

RESULTS

Effects of WA, CD, and CC on the Organization of the Cytoskeletal Systems

The alterations in cell morphology and reorganization of IFs, AFs, and MTs after drug treatments were followed by indirect fluorescence microscopy. Figure 1 shows the reorganization of the three major cytoskeletal systems after cells were respectively treated with 15 μM WA, 1 μM CD, and 1 μM CC for 1 h. In untreated cells, all three cytoskeletal systems exhibit as extended filamentous networks radiating from the nuclear membrane to the cytoplasmic membrane (Fig. 1a–c). In WA-treated cells, vimentin IFs were found to be collapsed and aggregated around the nucleus, actin molecules dispersed through out the cytoplasm, and MTs were relatively little affected (Fig. 1d–f). On the other hand, CD had no effect on the organization of vimentin IFs and MTs, but actin molecules were found to be dispersed in the cytoplasm in contrast to the filamentous appearance in the untreated cells (Fig. 1g–i). In CC-treated cells, MTs were disrupted onto the nucleus, actin molecules dispersed, but the IF network was much less affected (Fig. 1j–l). The effects of these drugs on the organization of the cytoskeletal systems are summarized in Table I. The data indicated that IFs and MTs can be, respectively, disrupted by treating cells with WA and CC. It is of importance to note that under our experimental conditions, AFs were the most fragile filaments which were found to be disassembled in cells treated with any of the above drugs (Table I).

Effects of WA, CD, and CC on the Development of Acquired Thermotolerance in 9L RBT Cells

Induction of thermotolerance in 9L RBT cells was achieved by a priming heat-shock treatment at 45°C for 15 min followed by a recovery period at 37°C for several hours. The development of acquired thermotolerance in the heat-primed cells was determined by the surviving fraction of the cells challenged with a lethal dose of heat-shock for 60 min at 45°C after the recovery period. As shown in Figure 2A, surviving fractions of the cells increased rapidly as the recovery duration lengthened from 0 to 4 h, and remained at this level for at least another 8 h. When compared to the cells which were challenged without a recovery period, 4 h of recovery led to a 200-fold (2.05×10^{-1} vs. 1.06×10^{-3}) increase in cell survival of the heat-primed cells. To investigate the possible involvement of the cytoskeletal systems in the development of acquired thermotolerance in the heat-primed cells, WA, CD, and CC were individually added either 45 min prior to the priming heat-shock which was carried out in the presence of the drug (pre-priming drug treatment), or immediately after the priming heat-shock for 60 min (post-priming drug treatment). At the end of the combined heat and drug treatment, cells were again allowed to recover in fresh medium under normal conditions for various durations and then challenged with a heat-shock dose at 45°C for 1 h. It was found that cells were unable to acquire thermotolerance when they were pre-priming treated with WA. The surviving fractions of cells treated with 15 μM WA for 45 min followed by a heat-shock at 45°C for 15 min in the presence of the drug were below 10^{-5}. In contrast, post-priming WA treatment resulted in a much higher cell survival (3.72×10^{-2} after 8 h of recovery), although it was still lower than the heat-primed control (Fig. 2B; Table I). In CD and CC treated cells, the differences in cell survival between pre- and post-priming drug treatments were negligible, both survival curves and maximal levels of thermotolerance acquired were similar to that of the heat-primed cells (Fig. 2C,D; Table 1). The data also demonstrated that 4 to 6 h of recovery was enough for the acquired

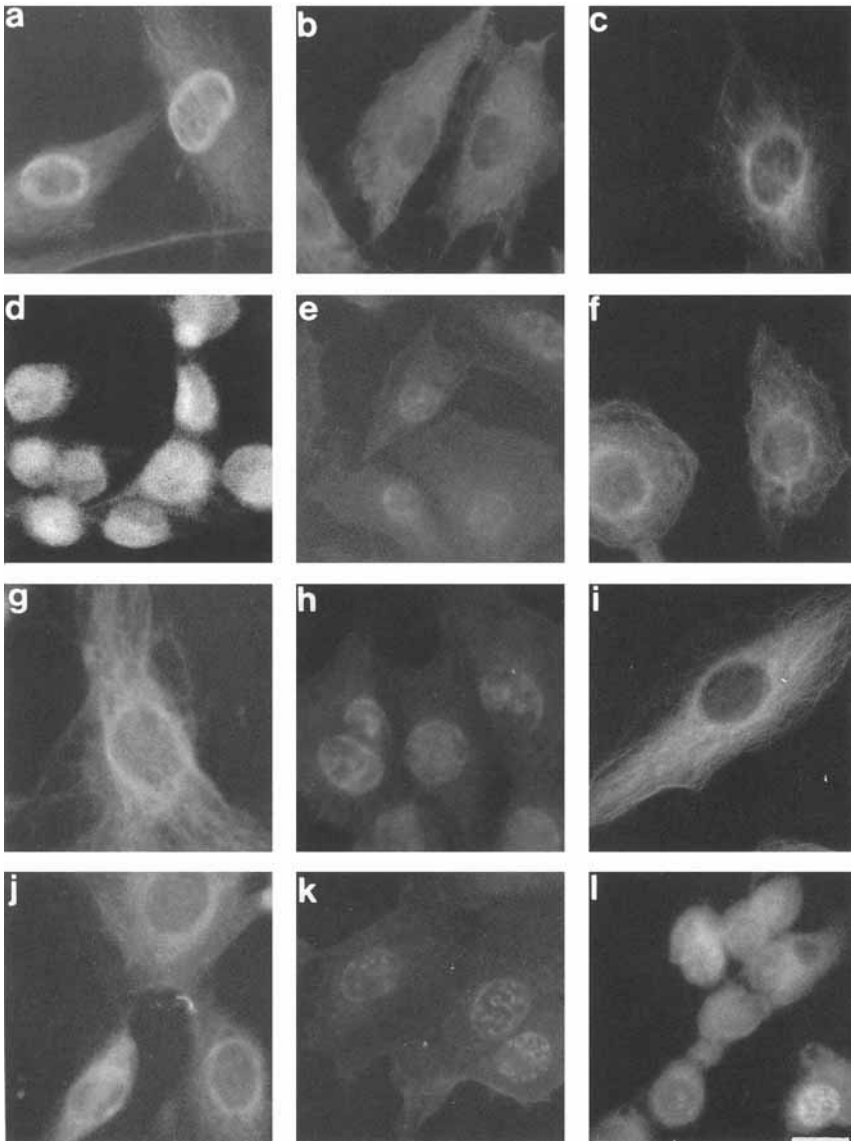


Fig. 1. Reorganization of the major cytoskeletal systems in 9L RBT cells treated with WA, CD, and CC. Cells were respectively treated with 15 μM WA (**d–f**), 1 μM CD (**g–i**), and 1 μM CC (**j–l**) for 1 h. After treatment, cells were fixed and processed for indirect immunofluorescence microscopy as described in Materials and Methods. Organizations of IFs, AFs, and MTs were revealed by using mouse monoclonal antibodies against vimentin (a,d,g,j), actin (b,e,h,k), and tubulin (c,f,i,l), respectively. (a–c) Untreated control cells. Bar, 5 μm.

thermotolerance developed to its maxima under all experimental protocols.

Effects of WA, CD, and CC on the Morphology of 9L RBT Cells

Morphological changes after 4 h of recovery in the above treatment protocols were determined by microscopic examination. When the cells were treated with a priming heat-shock and allowed to recover for 4 h, the morphology of these cells returned to their normal appearance (Fig. 3a,b). By contrast, cells subjected to a pre-priming WA treatment showed a rounded-up morphology after 4 h of recovery but there was only a slight morphological change in cells post-priming treated with WA (Fig. 3c,d). This observation was consistant with the effect of WA on the development of thermotolerance. In CD and CC treated cells, there was no significant difference in cell morphology between the pre- and post-priming drug treatments (Fig. 3e–h). These data showed that cells able to develop thermotolerance were also able to recover morphologically, and that the effect of WA treatment on the

TABLE I. Effects of Cytoskeletal Inhibitors on the Organization of the Three Major Cytoskeletal Systems and the Development of Acquired Thermotolerance in 9L RBT Cells

Drugs	Organization of the three major cytoskeletal systems			Maximal surviving fractions at the highest thermotolerant state ($\times 10^{-2}$)	
	IF	AF	MT	Pre-priming	Post-priming
No drugs	Filamentous	Filamentous	Filamentous	23.78 ± 4.45	
WA	Aggregated	Dispersed	Filamentous	0.00 ± 0.00	3.72 ± 0.07
CD	Filamentous	Dispersed	Filamentous	19.51 ± 5.52	23.46 ± 8.95
CC	Filamentous	Dispersed	Aggregated	16.62 ± 6.37	21.37 ± 8.15

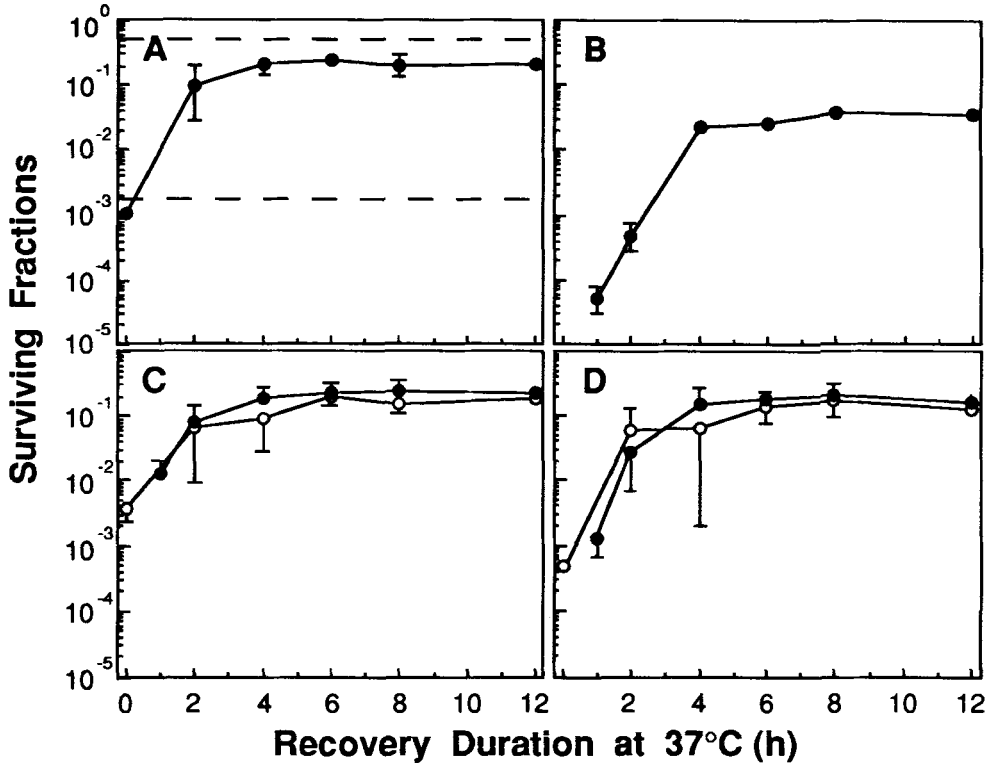


Fig. 2. Effects of the cytoskeleton inhibitors, WA, CD, and CC on the development of thermotolerance in 9L RBT cells. Thermotolerance was induced by a priming heat-shock at 45°C for 15 min followed by a recovery period as indicated (**A**). WA (**B**), CD (**C**), and CC (**D**) were added either 45 min prior to the priming heat-shock which was then carried out in the presence of the drugs (pre-priming drug treatments, open symbols) or immediately after the priming heat-shock and the cells were treated with the drugs for 1 h (post-priming drug treatments, closed symbols). Cells were allowed to recover at 37°C for various durations and then challenged with a second heat-shock at 45°C for 60 min. Surviving fractions were determined by colony formation assays and the data are the means of three independent experiments. In (A), the upper and lower dash lines represent cell survivals after heat-shock treatments at 45°C for 15 and 60 min, respectively. Data are means ± S.E. from three independent experiments.

development of thermotolerance was more prominent than the two other drugs, CD and CC.

Intracellular Distributions of Vimentin and HSC70 After Heat-Shock and Heat-WA Combined Treatments

The intracellular distributions of vimentin and HSC70 were monitored by immunofluorescence. In heat-shocked cells, vimentin IFs were found to be aggregated around the nucleus (Fig. 4a, compare with Fig. 4c). As the recovery duration increased, vimentin IFs were able to return to their filamentous appearance (Fig. 4d,g,j). However, in cells treated with WA before the priming heat-shock, vimentin IFs severely aggregated around the nucleus and remained unchanged for at least 8 h of recovery (Fig. 4b,e,h,k). It was

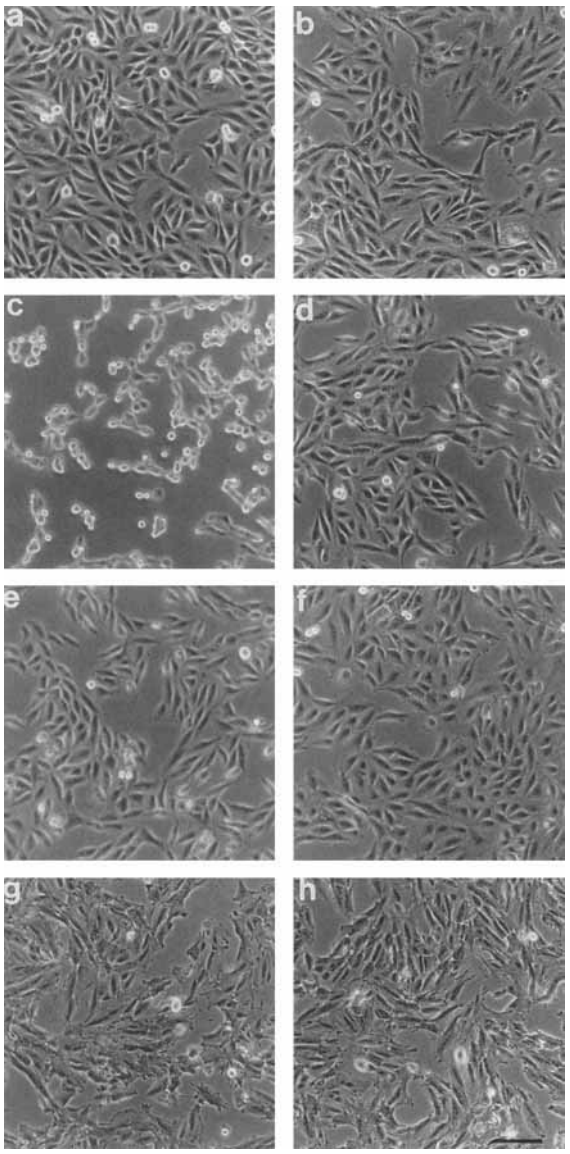


Fig. 3. Morphology of 9L RBT cells after recovering from heat-shock and heat-drug combined treatments. Cells were treated with a combination of heat and drugs as described in the legend of Figure 2. Cells were incubated at 37°C for 4 h after the priming heat-shock and then photographed. **a:** untreated controls; **b:** cells treated with the priming heat-shock only; **c,e,g:** cells were pre-priming treated with WA, CD, and CC, respectively; **d,f,h:** cells were post-priming treated with WA, CD, and CC, respectively. Bar, 30 μm.

also found that the redistribution of vimentin in cells treated with WA after the priming heat-shock (Fig. 4f,i,l) was similar to that of cells treated with heat-shock only (Fig. 4a,d,g,j). It is evident that addition of WA before heat-shock disrupted the integrity of vimentin. As a result, cell morphology was altered and the development of thermotolerance was abrogated. The redistributions of HSC70 after the above treatments were simultaneously followed. As shown in Figure 5, a portion of HSC70 gathered to the perinuclear position after recovering for 1 h from heat-shock treatment (Fig. 5d). It was observed that HSC70 migrated into the nucleus after 8 h of recovery (Fig. 5j). In cells pre-incubated with WA and then followed by a heat-shock treatment, HSC70 aggregated onto the nucleus, in a manner similar to vimentin, and this was also an irreversible event (Fig. 5b,e,h,k). However, when the drug was added after the heat-shock treatment, HSC70 first aggregated to the perinuclear position and then migrated into nucleus after 8 h of recovery from heat-shock (Fig. 5f,i,l), a behavior similar to that of the heat-primed cells.

Changes in Detergent Extractability of HSC70 After Heat-Shock and Heat-WA Combined Treatments

In addition to the immunofluorescence microscopic studies, redistribution of HSC70 in the above treatments was also determined by its changes in detergent extractability. Immunoblots are shown in Figure 6 and the corresponding quantitative changes are presented in Figure 7. In untreated cells, almost all HSC70 were detergent-extractable (Figs. 6A, 7A). It was found that a portion of HSC70 became non-extractable immediately after the priming heat-shock at 45°C for 15 min. As the recovery time increased, the non-extractable HSC70 returned to being extractable (Figs. 6A, 7A). The data also showed that the inducible HSP70 rapidly accumulated during the recovery, and that this protein was totally soluble in our extraction protocol (Fig. 6A). As shown in Figures 6B and 7B, treatment with 15 μM WA for 60 min also resulted in the reduction in extractability of HSC70 which partitioned into the non-extractable fraction gradually. In cells treated with WA before or after heat-shock, just as in the heat-shocked cells, a portion of HSC70 became non-extractable (Figs. 6C,D, 7C,D). However, these non-extractable HSC70 did not return to the extractable fraction after 12 h of recovery. It was also found that the total amounts of HSC70 increased in cells subjected to all treatment protocols (Fig. 7).

DISCUSSION

We previously reported that WA, a novel withanolide isolated from the antitumor Chi-

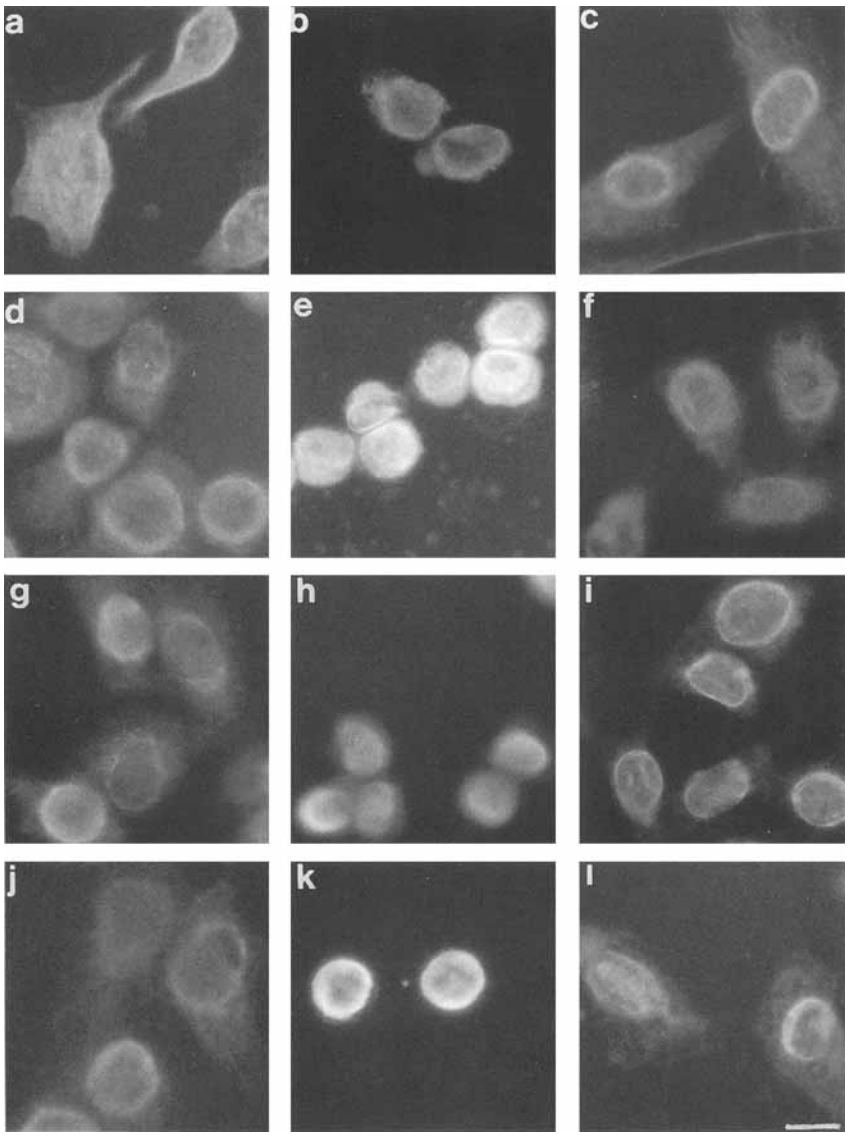


Fig. 4. Intracellular distribution of vimentin in heat-primed 9L RBT cells and in cells pre- and post-priming treated with WA. Cells were heat-primed **(a,d,g,j)**, pre- **(b,e,h,k)**, or post-priming **(f,i,l)** treated with 15 μM of WA and allowed to recover from the priming heat-shock for 0 (a,b), 1 (d–f), 4 (g–i), and 8 h (j–l) as described in Materials and Methods as well as in the legend of Figure 2. Intracellular distribution of vimentin was analyzed by indirect immunofluorescence microscopy using mouse monoclonal antibodies to vimentin as a probe. (c) Untreated controls. Bar, 5 μm.

nese herb *Physalis angulata,* may function as a cytoskeleton inhibitor [Lee et al., 1993]. After treatment with 50 μM WA for 1 h (S.F. $\leq 10^{-2}$), all three cytoskeletal systems in 9L RBT cells were found to be collapsed or disrupted. In particular, the drug can elicit a number of biochemical changes of the vimentin protein [Lee et al., 1993]. In this study, however, we have demonstrated that WA turns out to be a relative vimentin IF-specific drug at a lower concentration. In cells treated with 15 μM WA for 1 h (S.F. 4×10^{-1}), IFs were collapsed and clustered around the nucleus in 9L RBT cells. Under this treatment condition, AFs were also disrupted but MTs were little affected. We have also shown that AFs were also destructed in CD- and CC-treated cells.

In heat-shocked cells, vimentin IFs are found to have collapsed and aggregated around the nucleus [Welch and Suhan, 1985; Collier and Schlesinger, 1986; Collier et al., 1993; Cheng and Lai, 1994], AFs are disrupted [Wiegant et al., 1987], and MTs networks are disassembled or collapsed [Wiegant et al., 1987; van Bergen en

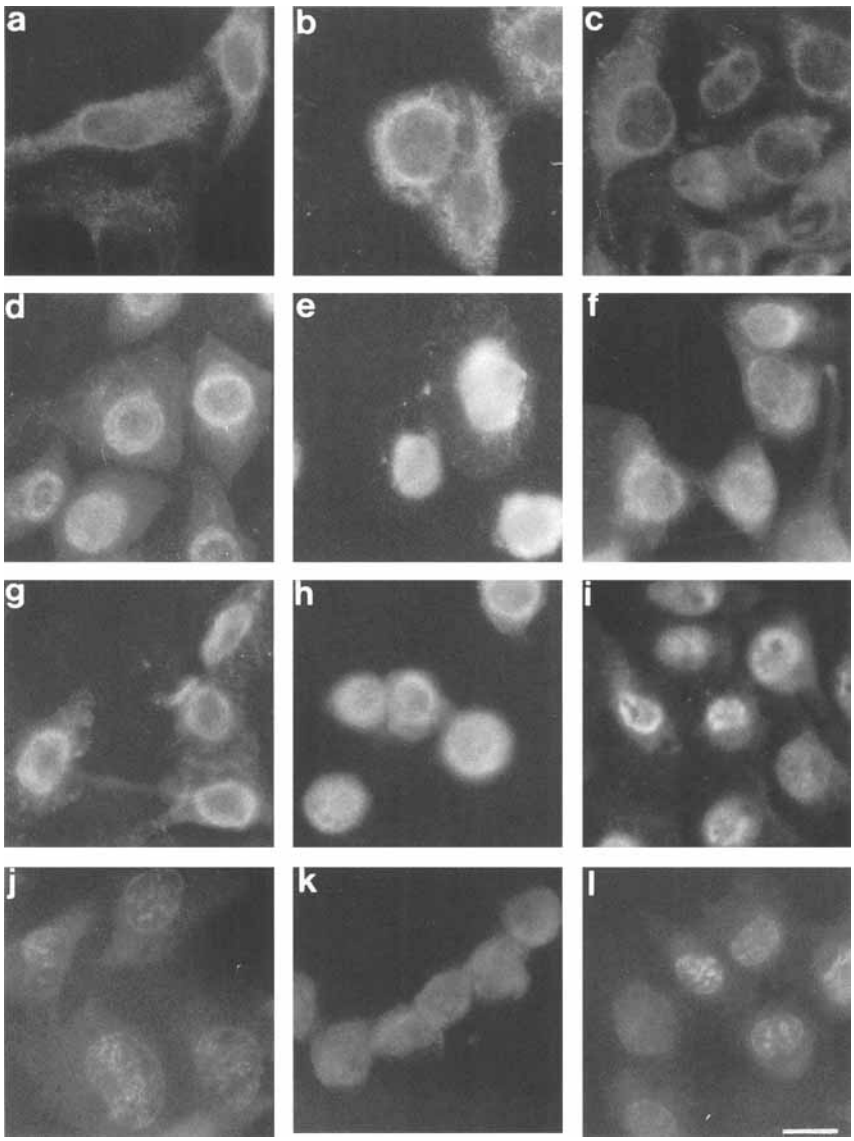


Fig. 5. Intracellular distribution of HSC70/HSP70 in heat-primed 9L RBT cells and in cells pre- and post-priming treated with WA. Cells were heat-primed (**a,d,g,j**) pre- (**b,e,h,k**), or post-priming (**f,i,l**) treated with 15 μM of WA and allowed to recover from the priming heat-shock for 0 (a,b), 1 (d–f), 4 (g–i), and 8 h (j–l) as described in the Materials and Methods as well as in the legend of Figure 2. Intracellular distribution of HSC70/HSP70 was analyzed by indirect immunofluorescence microscopy using mouse monoclonal antibodies to HSC70/HSP70 as a probe. (c) Untreated controls. Bar, 5 μm.

Henegouwen et al., 1985]. If the cells are allowed to recover from the initial heat-shock treatment, the entire cytoskeleton will return to the original organization in a process that usually requires several hours. Most interestingly, acquired thermotolerance is developed at the same time. Therefore it has been suggested that the integrity of cytoskeleton may be responsible for the development of thermotolerance [Welch and Mizzen, 1988]. In this study, WA, CD, and CC were used to dissect the relationship between the individual cytoskeletal systems and the development of thermotolerance in cells treated with a sublethal (priming) dose of heat-shock. Individual cytoskeleton inhibitors were added either before or after the priming heat treatment and the development of thermotolerance was subequently monitored. The data showed that cells became thermotolerant when they were recovering from the priming heat treatment and the development of thermotolerance was not affected by the presence of CD and CC, indicating that AFs and MTs might not be involved in this process. In contrast, in cells

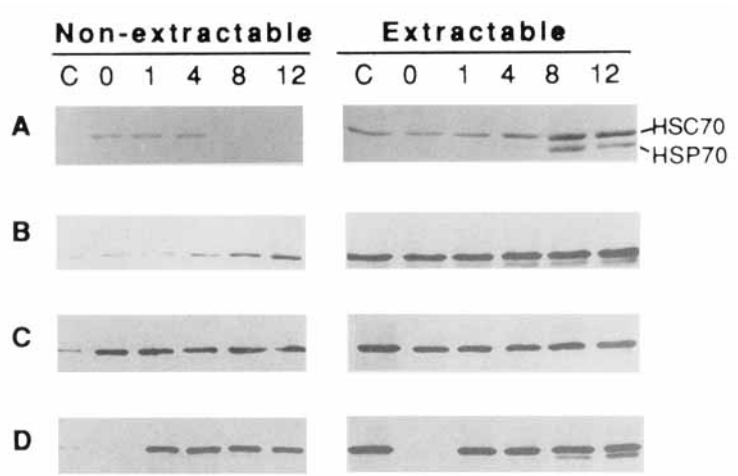
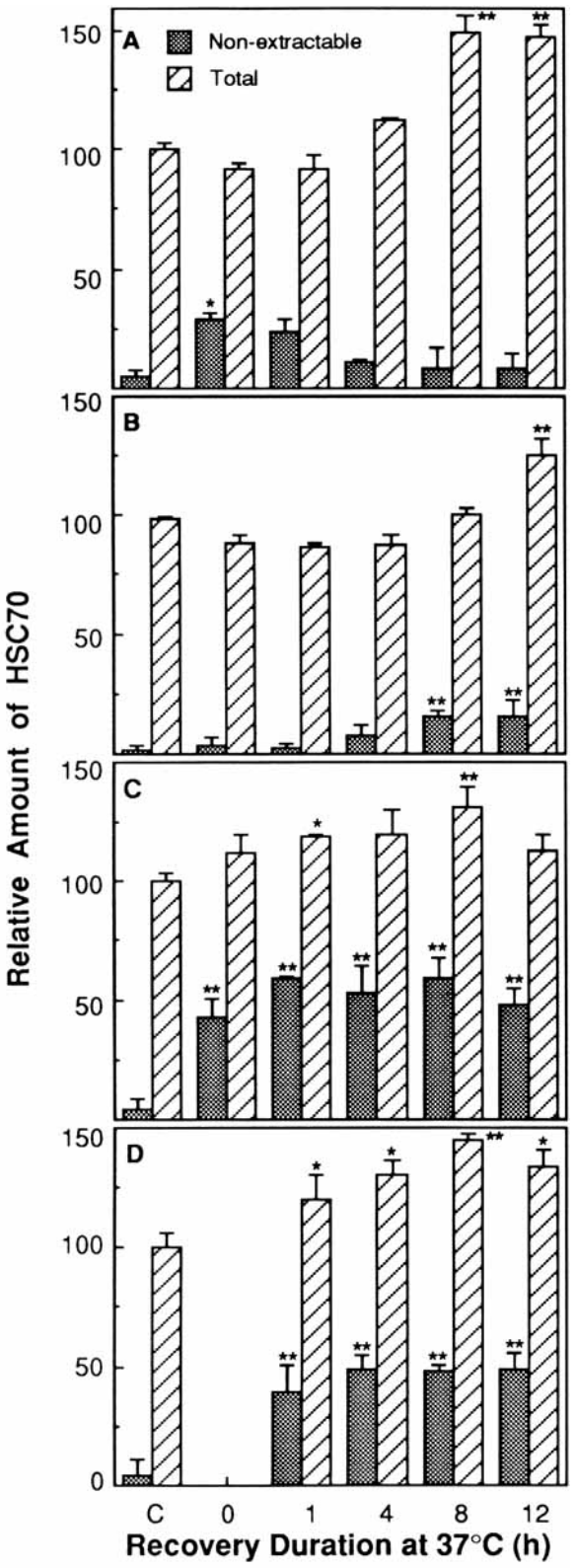


Fig. 6. Detergent extractability of HSC70/HSP70 in heat-primed and WA-treated 9L RBT cells as well as in cells pre- and post-priming treated with WA. Cells were (**A**) heat-shocked at 45°C for 15 min, (**B**) treated with 15 μM WA for 60 min, (**C**) pre-priming treated with 15 μM WA for 45 min followed by heat-shocked at 45°C for 15 min in the presence of the drug, or (**D**) heat-shocked at 45°C for 15 min followed by treatment 15 μM WA for 60 min at 37°C. After treatment, cells were allowed to recover from the priming heat-shock for 0, 1, 4, and 8 h as indicated by the numbers on the tops of the lanes. The cells were then lysed with extraction buffer for 10 min and the cellular proteins were collected as extractable and non-extractable fractions. The samples were resolved by SDS-PAGE and the gels were processed for immunoblotting by using mouse mAb to HSC70/HSP70 as a probe. Lane C, untreated controls.

treated with WA and then the priming heat-shock, the development of thermotolerance was completely abrogated and no cells survived when they were subsequently subjected to the challenge dose of heat-shock treatment. However, when WA was added after the priming heat treatment, cells were able to acquire thermotolerance to a lesser extent when compared to the heat-primed cells. Taken together, these observations strongly suggested that the integrity of IFs may relate to the development of acquired thermotolerance in heat-shocked cells (Table I). Interestingly, it has been reported that increased amounts of the vimentin-containing IFs are correlated with the heat-resistant phenotypes in Chinese hamster ovary cells [Lee et al., 1992a].

As mentioned previously, HSPs have been suggested to be associated with the acquisition of thermotolerance in heat-shocked cells [Li and Werb, 1982; Landry et al., 1982; Laszlo and Li, 1985; Chretien and Landry, 1988; Sanchez and Lindquist, 1990; Hahn and Li, 1990]. Specific co-localizations of members of HSPs and cytoskeletal components have been demonstrated by in vitro and in vivo studies [Reiter and Penman, 1983; Nishida et al., 1986; Ohtsuka et al., 1986; Lee et al., 1992]. For instance, HSP27, 90, and 100 are found to be associated with actin [Koyasu et al., 1986; Lavoie et al., 1993]; HSC70 is involved in the assembly of microtubules [Gupta, 1990]; HSP70, the inducible form of the HSP70 family, is found to be complexed with tubulins in cells recovering from heat-shock [Lee et al., 1992]. In the present study, the intracellular distribution of HSC70, the constitutively expressed member of HSP70, as well as the organization of vimentin IFs, were simultaneously monitored by immunofluorescence staining and detergent-extractability studies. In cells pre-priming treated with WA, vimentin IFs were irreversibly disrupted and the HSC70 was irreversibly associated with the aggregated IFs as indicated by indirect immunofluorescence as well as by an irreversible decrease in detergent-extractability of the HSC70 molecules. The data indicated that in cells pre-priming treated with WA, the damage of IFs caused by WA was too severe to be repaired by the binding of HSC70. On the other hand, in cells post-priming treated with WA, the vimentin IFs appeared to be relatively normal after 8 h or recovery. The data indicated that during the priming heat treatment, the IFs were stabilized by, possibly, binding with HSC70, and the cells were able to recover as well as to acquire thermotolerance during the course of recovery. The data further suggested that binding to HSC70 to the IFs elicited by the priming heat treatment, was able to diminish the adverse effect of WA.

It was previously reported that a significant portion of HSC70/HSP70 accumulated within



the nucleus and, in particular, the nucleolus soon after heat shock treatment [Pelham, 1984; Velazquez and Lindquist, 1984; Welch and Feramisco, 1984; Kampinga et al., 1993]. Analysis of the nucleolus after heat shock by high-resolution electron microscopy has revealed significant amounts of denatured and aggregated preribosomal components [Welch and Suhan, 1985]. In this study, it was shown that a portion of HSC70 became non-extractable and gathered to the perinuclear soon after heat shock but migration into the nucleus could be detected at earliest after 8 h of recovery. Therefore, a significant amount of HSC70 that migrated into the nucleus could be available only after most of the denatured vimentin had recovered from aggregation. The binding of HSC70 to the disrupted vimentin IFs may participate in the transactivation of heat-shock genes [Lee et al., 1991, 1993; Cheng and Lai, 1994]. It has been suggested that abnormal or denatured proteins are responsible for the induction of HSPs synthesis [Ananthan et al., 1986; Lee and Hahn, 1988; Edington et al., 1989; Hightower, 1991; Burgman and Konings, 1992]. Also, HSC70 has been shown to be associated with a number of presumed denatured proteins during or after stress treatment [Collier and Schlesinger, 1986; Napolitano et al., 1987; Dubois et al., 1989]. More recently, it has been suggested that HSC70 may complex with HSF and act as a heat sensor involved in the regulation of the heat-shock genes [Craig and Gross, 1991; Abravaya et al., 1992]. Since vimentin is abundant in 9L RBT cells, it is possible that vimentin is one of the major denatured proteins to be chaperoned by HSC70 after heat-shock treatment. If the affinity of disrupted vimentin IFs with HSC70 is higher than that of normally complexed proteins, HSC70 would dissociate from the normal protein complexes and result in the activation of the heat-shock factor and then the transactivation of heat-shock genes.

In summary, our observations lead us to conclude that the integrity of IFs may be important for the development of thermotolerance in cells

Fig. 7. Quantitative changes in detergent-extractability of HSC70 in heat-primed and WA-treated 9L RBT cells as well as in cells pre- and post-priming treated with WA. Typical immunoblots as shown in Figure 6 were quantitated by scanning densitometry. Panel labels (**A–D**) shown here are the same as in Fig. Data are means ± S.E. from three independent experiments. *Indicate significant differences when compared to controls, $P < 0.05$; **, $P < 0.01$.

recovering from a priming heat-shock treatment.

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