Journal of Cellular Biochemistry 52:253–265 (1993)

ARTICLES

Induction of Vimentin Modification and Vimentin-HSP72 Association by Withangulatin A in 9L Rat Brain Tumor Cells

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Abstract Withangulatin A induced cell rounding up and the morphological alteration resulted from the reorganization of all of the major cytoskeletal components, i.e., vimentin, tubulin, and actin, as revealed by immunofluorescence techniques. When the withangulatin A-treated cells changed to a round-up morphology, vimentin intermediate filaments were found to be collapsed and clustered around the nucleus. The alteration was accompanied by characteristic changes of vimentin molecules, including augmentation of phosphorylation, retardation of electrophoretic mobility, and decrease in detergent extractability. The levels of vimentin phosphorylation were augmented by 2.5- and 1.8-fold in cells incubated with 50 μM withangulatin A for 1 and 3 h, respectively. The electrophoretic mobility of vimentin was partially retarded in cells treated with withangulatin A for 1 h at 10 μM and a completely upshift mobility was observed after 5 h treatment at 50 μM. In addition, vimentin molecules became less extractable by nonident P-40 after the cells were treated with withangulatin A and this effect was dose dependent. The decrease in solubility of vimentin was accompanied by the redistribution of HSP72 into the detergent nonextractable fraction and these two events were well correlated. Our results suggest that withangulatin A induced the modification of vimentin, which resulted in the alteration of cell morphology and redistribution of intracellular HSP72, an event that may play an important role in the induction of heat-shock response.

Key words: heat-shock proteins, stress response, vimentin, intermediate filament, withangulatin A

Cytoskeleton is an extensive and intricate protein scaffold in the cytoplasm. 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]. Among the major cytoskeletal components, intermediate filaments appear to be the most stable fibrils of mammalian cells. There are at least five distinct classes of proteins making up the various intermediate filaments [for reviews see Steinert and Roop, 1988; Bloemendal and Pieper, 1989]. The most widely distributed class of intermediate filaments are composed of a single subunit protein called vimentin in cells of mesenchymal origin and most cells in culture. Vimentin intermediate filaments form a network which makes contact with the nuclear envelope and the plasma membrane [Lazarides, 1980; Georgatos and Blobel, 1987], thus forming an elaborate system of nucleolemmal/plasmalemmal interactions [Geiger, 1987; Skalli and Goldman, 1991]. An interesting feature of vimentin is the presence of a small proportion as a phosphorylated variant [Cabral and Gottesman, 1979; Nelson and Traub, 1982]. Two-dimensional gel analysis of proteins from ^{32}P-labeled cells has revealed that phosphorylated vimentin is among the most prominent phosphoproteins in the cytoplasm [O'Connor et al., 1981; Spruill et al., 1983; Isaacs et al., 1989; Lee et al., 1992]. The phosphorylation of vimentin has been demonstrated to be site specific and restricted to the 9 kDa N-terminal domain [Inagaki et al., 1987; Geisler et al., 1989; Ando et al., 1989]. Although phosphorylation of vimentin appears to be associated with disassembly of vimentin filament [Lamb et al., 1989; Chou et al., 1989; Isaacs et al., 1989; Inagaki et al., 1987; Geisler et al., 1989; Ando et al., 1989; Wyatt et al., 1991], its detail physiological function is not completely

Received December 9, 1992; accepted January 7, 1993.

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understood at present. Nevertheless, it has been shown that vimentin organization in cells is altered dramatically during mitosis [Franke et al., 1984; Lamb et al., 1989; Chou et al., 1989] and heat-shock [Thomas et al., 1982; Welch and Suhan, 1985], as well as in the presence of protein phosphatases inhibitors [Chartier et al., 1991; Lee et al., 1992].

Heat-shock proteins (HSPs), induced in cells subjected to supraoptimal temperature and other related physiological insults, are highly conservative and usually identified by their apparent molecular weights [for review see Ashburner and Bonner, 1979; Burdon, 1986; Lindquist, 1986; Schlesinger, 1990]. Three classes of major HSPs, i.e., HSP70, 90, and 110 are commonly detected in mammalian cells [Lindquist and Craig, 1988]. HSP70 family in rat cells has been reported to be consisted of at least two members: HSP72, a constitutive form (sometimes referred as HSC70) and HSP70, an inducible form [Hightower and White, 1981; Lee et al., 1991]. HSP72 can associate with a variety of proteins under normal conditions to perform functions such as integrating cytoskeletal organization [Koyasu et al., 1986; Ahmad et al., 1990], uncoating clathrin triskelon [Chappell et al., 1986; Deluca-Flaherty et al., 1990], inactivating protein kinases, and steroid hormone receptor [Schlesinger, 1990; Sanchez et al., 1990], as well as binding and transporting newly synthesized proteins [Deshaies et al., 1988; Ellis and Hemmingsen, 1989; Beckmann et al., 1990; Scherer et al., 1990]. Moreover, HSP72 has also been shown to precipitate with other cellular proteins (presumably denatured) during or after stress treatment [Collier and Schlesinger, 1986; Napolitano et al., 1987; Dubois et al., 1989]. Thus, it is conceivable that the level of homeostatic HSP72 in normal growth conditions is reduced after stress treatment and by which the stress response is triggered [Schlesinger et al., 1990; Sorger, 1991; Craig and Gross, 1991].

Withangulatin A, a novel compound isolated from the Chinese antitumor herb *Physalis angulata* [Chen et al., 1990], has been identified as a topoisomerase II inhibitor in vitro [Juang et al., 1989]. In vivo, it is found to be cytotoxic, capable of suppressing general protein synthesis, and inducing the synthesis of HSPs [Lee et al., 1991]. Since the cellular effects of withangulatin A are similar to those of heat treatment, we proposed that withangulatin A may cause protein denaturation so that HSPs synthesis can be induced [Lee et al., 1991]. To investigate the mechanism underlying the stress response, the cellular and biochemical events affected by withangulatin A were further characterized. In the present study, we found that the cell morphology was altered and vimentin was specifically modified by withangulatin A in several ways. Furthermore, both vimentin and HSP72 were found to be redistributed together in the detergent nonextractable fractions after the cells were treated with withangulatin A. Such observations suggest that the modification of vimentin by withangulatin A may play a role in the induction of stress response.

MATERIALS AND METHODS

Materials

Withangulatin A was isolated from *Physalis angulata* as described [Chen et al., 1990]. It was dissolved in dimethyl sulfoxide at a concentration of 20 mM, stored in the dark at 4°C and diluted to appropriate concentrations with culture medium before use or directly added to the medium to the final concentrations. All cultureware was obtained from Corning (Corning, NY) and culture medium components were purchased from GIBCO (Gaithersburg, MD). [^{35}S]methionine (specific activity >800 Ci/mmole) and [^{32}P]orthophosphate (specific activity 8,500–9,120 Ci/mmole) were obtained from New England Nuclear (Boston, MA). Supplies for immunoblotting and immunofluorescence were purchased from Amersham (Buckinghamshire, England) or BioRad (Richmond, CA). Monoclonal antibodies for the cytoskeletal components and HSP72 were from Amersham and StressGen (Victoria, BC, Canada), respectively. Chemicals for electrophoresis were obtained from BioRad. General chemicals were supplied by Sigma (St. Louis, MO) or Merck (Darmstadt, Germany).

Cell Culture and Drug Treatment

The 9L brain tumor cells, originated from rat gliosarcoma, was a generous gift from Dr. M.L. Rosenblum, University of California at San Francisco [Weizsaecker et al., 1981]. Cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin. The cells were kept in a humidified 37°C incubator with a mixture of 5% CO_2 and 95% air. Stock cells were plated in six-well plates

at a density of 4 to 6 × 10^4 cells per cm^2. All experiments were performed using exponentially growing cells at 80–90% confluency. For the studies of drug effects, cells were treated with various concentrations of withangulatin A at 37°C for various durations as indicated.

Indirect Immunofluorescence and Microscopy

For indirect immunofluorescence studies, cells were grown on chamber slides (Nunc) and treated with withangulatin A as indicated. 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 30 min at room temperature with monoclonal antibodies against vimentin, tubulin, and actin (diluted 1:20, 1:50, and 1:50, respectively). The antibodies were diluted in PBS containing 3% BSA and their specificity were pretested by immunoblotting techniques. The cells were then washed with PBS and incubated with fluorescein-conjugated goat anti-mouse IgG for 20 min at 1:20 dilution. 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.

In Vivo [^{32}P]phosphate Labeling of Phosphoproteins

To study the level of phosphorylation of vimentin, cells were prelabeled with 1 mCi of [^{32}P] orthophosphate for 1 h in 1 ml of phosphate-free medium prior to the drug treatment. Withangulatin A was added to the labeling medium to the final concentration of 50 μM and the cells were further incubated for 1 or 3 h. After treatments, the cells were washed, lyzed, and subjected to electrophoresis as described in the followings. For the visualization of ^{32}P-labeled proteins, the gels were fixed, dried, and directly proceeded for autoradiography. Films were exposed at room temperature in the presence of an intensifying screen.

Gel Electrophoresis and Autoradiography

After drug treatments, the cells were briefly washed with ice-cold phosphate buffered saline (PBS) and lyzed with 300 μl of sample buffer (0.0625 M Tris-HCl, pH 6.8; 2% sodium dodecylsulfate, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) or 200 μl lysis buffer (9.5 M urea, 2% nonident P-40, 2% ampholytes, and 5% β-mercaptoethanol) depending on the electrophoresis system employed. One-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970]. The samples for SDS-PAGE were heated in boiling water for 5 min and then microfuged (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 molecular weight calibration, a subset of the following standards (Sigma Chemical Co., St. Louis, MO) were included in each gel: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). After electrophoresis, the gels were removed, stained for 1 h in staining solution (0.1% Coomassie brilliant blue R250 in 10% acetic acid and 50% methanol). The gels were then destained and dried under vacuum. Autoradiography was performed at room temperature using Fuji RX X-ray film. Alternatively, the gels were processed for immunoblot analysis. Two-dimensional PAGE was performed according to the method of O'Farrell [1975]. Equal amount of cell lysates were loaded onto the pre-run isoelectrofocusing (IEF) gels and run for 16 h at 400 V and then 1 h at 800 V. Subsequently, the IEF gels were loaded onto 10% SDS-polyacrylamide slab gels with a 4.75% stacking gel for electrophoresis in the second dimension. After electrophoresis, the gel slabs were processed for Coomassie blue staining, autoradiography, or immunoblot analysis. The pH gradient formed was measured from slices obtained from replicate IEF gels.

Immunoblot Analysis

After electrophoresis, the gel was soaked in transfer buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) for 10 min. Resolved proteins were then electro-transferred onto a nitrocellulose membrane (Hybond-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 20 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 vimentin or HSP72 (diluted 1:200

or 1:200 in TTBS containing 1% gelatin, respectively) at room temperature for 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).

Protein Extraction and Cell Fractionation

Cellular proteins were separated into "extractable" and "nonextractable" fractions before they were subjected to gel electrophoresis as described. Cells were prelabeled with 30 to 50 μCi of [^{35}S]methionine for 6 h in 1 ml of methionine-free culture medium and then treated with withangulatin A as described above. After drug treatments, the cells were washed once with room temperature PBS and incubated with 0.2 ml of extraction buffer (1% nonident P-40 and 0.15 M NaCl in 50 mM Tris-HCl, pH 8.0) on ice for another 30 or 60 min. Soluble proteins extracted under this condition were collected into microfuge tubes and the samples were centrifuged (Eppendorf, full speed) at 4°C for 10 min. Aliquots of the supernatants, referred as the extractable fraction of cellular proteins, were added to equal amounts of double-strength sample buffer before electrophoresis. On the other hand, cellular proteins remained 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 as the non-extractable fraction of the cellular proteins and presumably to be the nuclear-cytoskeletal fraction as previously reported [Collier and Schlesinger, 1986; Lee et al., 1992].

RESULTS

Effects of Withangulatin A on Cell Morphology and Redistribution of the Cytoskeletal Components

In the presence of withangulatin A, the cells rounded up as they were entering the mitotic phase. When the cells were exposed to the drug at the concentration of 50 μM, the morphological change was initially observable after 0.5 h and progressively became evident as the exposure time increased to 1 h (Fig. 1A–C). After 3 h, most of the cells rounded up from the culture matrix (Fig. 1D). A fraction of the cells ultimately detached from the culture vessel as the incubation time was prolonged beyond 5 h (data not shown). Since cell morphology is apparently maintained by the cytoskeleton, the redistribution of its components was explored by using indirect immunofluorescence microscopy. It was found that after 1 h treatment of withangulatin A at 50 μM, the distribution of the major cytoskeletal components, including vimentin, tubulin, and actin were altered. Most significantly, the normally well-spread vimentin-containing intermediate filaments were found to be collapsed and clustered around the nucleus (Fig. 2A,D). On the other hand, tubulin and actin became diffusively distributed into the cytoplasm, in contrast to their well-organized structures found in the untreated cells (Fig. 2).

Effect of Withangulatin A on Vimentin Phosphorylation

Figure 3 showed that the level of vimentin phosphorylation was augmented after incubation of 50 μM withangulatin A. In control cells (Fig. 3A), the authentic vimentin was shown to be the major phosphoprotein whereas the degradation products were less phosphorylated (VDP1, 2, and 3) or unphosphorylated (VDP4). The level of phosphorylation of authentic vimentin was increased by 2.5-fold in cells incubated with 50 μM withangulatin A for 1 h. In contrast, this level is increased by 1.8-fold in cells incubated for 3 h. The levels of phosphorylation in VDP1 to 3 appeared to be augmented in 1 h treated cells but were less phosphorylated in 3 h samples. In addition, it was found that the phosphate moieties on these proteins were not stable under alkaline conditions (data not shown), indicating that the phosphorylation of vimentin was mediated by kinase(s) which phosphorylated the serine and/or threonine residues.

Effect of Withangulatin A on the Electrophoretic Mobility of Vimentin

In withangulatin A-treated cells, vimentin was found to be modified in a way that its electrophoretic mobility on SDS-polyacrylamide gels was retarded (Fig. 4). The protein showed an upshift

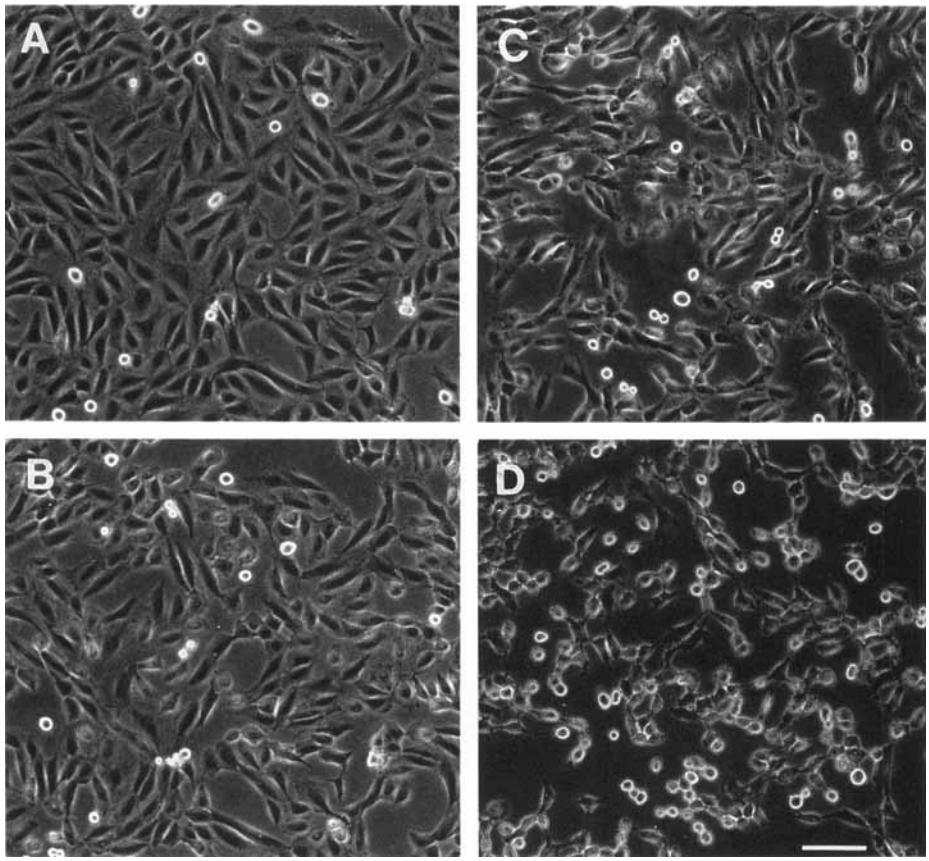

Fig. 1. Morphological changes of 9L cells after exposed to withangulatin A. Cells were incubated with 50 μM of withangulatin A for various durations before photographed. **A,** control cells; **B,** cells treated with drug for 0.5 h; **C,** cells treated for 1 h; **D,** cells treated for 3 h. Bar represents 50 μm.

of apparent molecular mass by approximately 2 kDa and the effect was vimentin specific. When the cells were incubated with 50 μM of the drug for 1 h, it appeared that half of vimentin was modified; after 5 h, practically all vimentin molecules existed as the modified form (Fig. 4A). Both of the increased levels of the modified vimentin and decreased amounts of the authentic vimentin appeared to be proportional to the incubation durations. When the cells were treated with different concentrations of withangulatin A, the modified form of vimentin was detected in the cells incubated with 10 μM withangulatin A for 1 h (Fig. 4B). After the same treatment duration, this modification became evident at the concentrations through 25 to 100 μM (Fig. 4B). This effect was further analysed by two-dimensional gel electrophoresis. Figure 5A demonstrated that the levels and mobilities of authentic vimentin (V) and its degradation products (VDPs) were affected by withangulatin A while that of actin and HSP72 were not changed. Owing to the slight difference in apparent molecular masses between the original and the modified forms, the retarded effect of mobility in authentic vimentin was not possible to be distinguished. However, the changes in mobility of VDPs, especially for VDP3 and VDP4, were clearly observed (Fig. 5A). Immunoblots showed in Figure 5B clearly demonstrated mobility retardations of VDPs after withangulatin A treatments. After 1 h treatment at 50 μM, both VDP3 and VDP4 were each equally split into two forms: upper (modified) and lower (original) forms. Moreover, 3 h treatment of withangulatin A resulted in higher level of modified VDP3 and completely modified VDP4.

Effect of Withangulatin A on the Detergent Extractability of Vimentin

The changes in detergent extractabilities of cellular proteins in withangulatin A-treated sam-

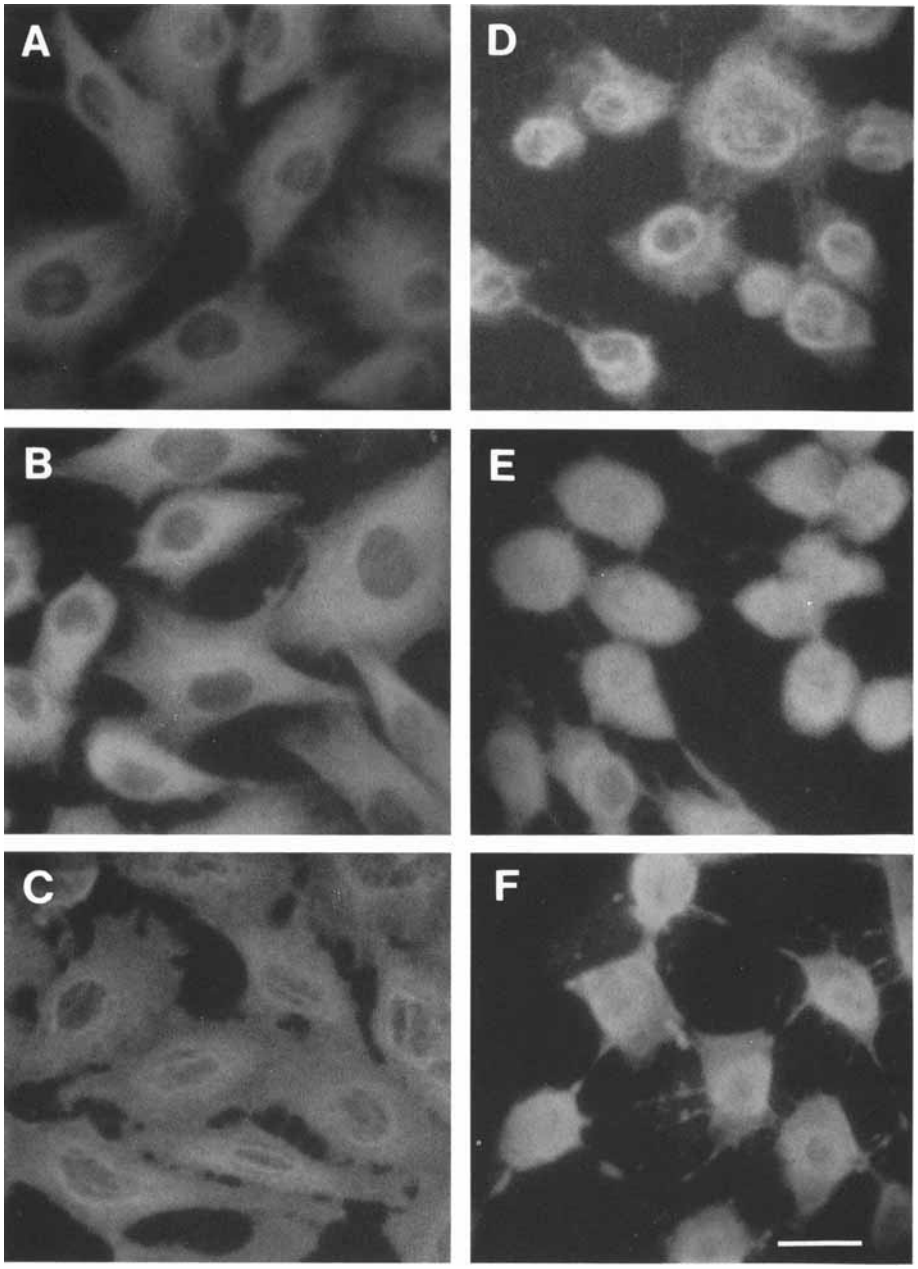

Fig. 2. Redistribution of vimentin, tubulin, and actin in cells treated with 50 μM of withangulatin A for 1 h. The control (**A–C**) and treated (**D–F**) cells were processed and analyzed by indirect immunofluoresence using mouse monoclonal antibodies against vimentin (A,D), tubulin (B,E), and actin (C,F), respectively. Bar represents 20 μm.

ples were analyzed by using cells that were metabolically labeled with [^{35}S]methionine. In spite of the durations of extraction, the extractability of actin and tubulin remained unchanged as in the untreated cells when the cells were lyzed with buffer containing 1% NP-40 and 0.15 M of NaCl. In contrast, the extractability of vimentin was found to be related to the extraction durations. In the 30 min extraction protocol, the extractability of vimentin decreased after the cells were treated with withangulatin A and the effect was a dose dependent. When the cells were treated with different concentrations of withangulatin A for 1 h, the amount of vimentin

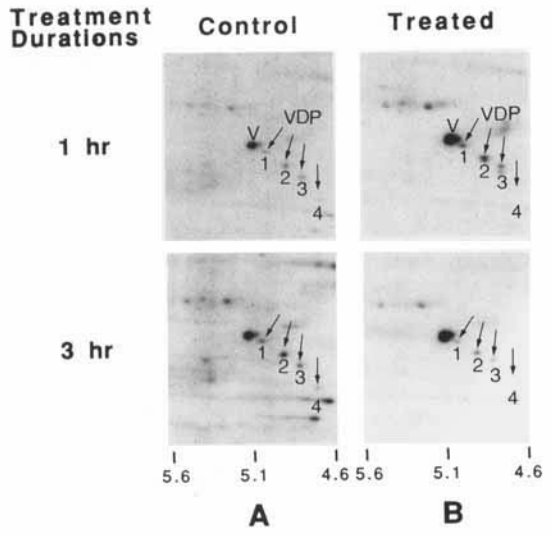

Fig. 3. Effect of withangulatin A on the phosphorylation state of vimentin in 9L cells. Cells were prelabeled with [^{32}P]phosphate and then incubated in the absence (**A**) or presence (**B**) of 50 μM of withangulatin A for 1 or 3 h. After treatments, the cells were lysed and the lysates were resolved by two-dimensional gel electrophoresis. The gels were then processed for autoradiography. Only the regions nearby vimentin and its degradation products are shown. V, authentic vimentin; VDP, degradation products of vimentin, numbers indicate the locations of the four primary VDPs. The pH range is shown at the bottom of the panels.

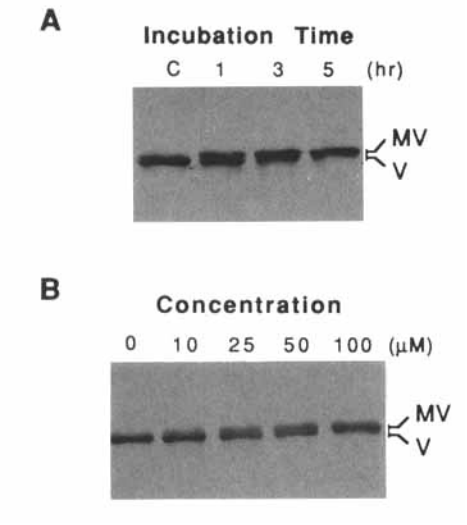

Fig. 4. Dose-dependent changes of electrophoretic mobility of vimentin induced by withangulatin A in 9L cells. Cells were incubated with 50 μM of withangulatin A for various durations (**A**) or with various concentrations of withangulatin A for 1 h (**B**). After treatments, the cells were lysed with sample buffer and the lysates were resolved by SDS-PAGE. Vimentin was visualized by immunoblotting techniques. C, control cells; V, vimentin; MV, modified vimentin.

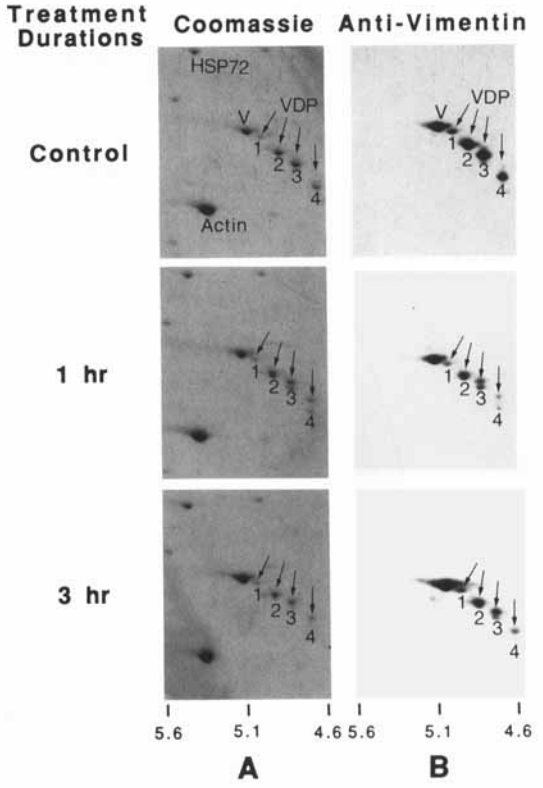

Fig. 5. Two-dimensional gel analysis of withangulatin A-induced changes in the electrophoretic mobility of vimentin and its degradation products. Cells were incubated with 50 μM of withangulatin A for 1 or 3 h. After treatments, the cells were lysed and the lysates were resolved by two-dimensional gel electrophoresis. The gels were then stained with Coomassie blue (**A**) and proceeded for immunoblotting (**B**). Only the regions nearby vimentin and its degradation products are shown. V, authentic vimentin; VDP, degradation products of vimentin, numbers indicate the locations of the four primary VDPs; HSP72, constitutive heat-shock protein with molecular mass of 72,000. The pH range is shown at the bottom of the panels.

partitioned into the nonextractable fraction was proportional to the drug concentrations from 0 to 25 μM and then leveled off (Fig. 6). On the other hand, when the cells were incubated with 50 μM of the drug, the amount of vimentin partitioned into the nonextractable fraction was proportional to the treatment duration. It was found that the decrease of extractable vimentin was detectable after 5 min and completed after 10 min of treatment (Fig. 7). The above results indicated that change in the detergent extractability of vimentin was a dose-dependent process and this process occurred in the dose ranges that the heat-shock response can be elicited by withangulatin A.

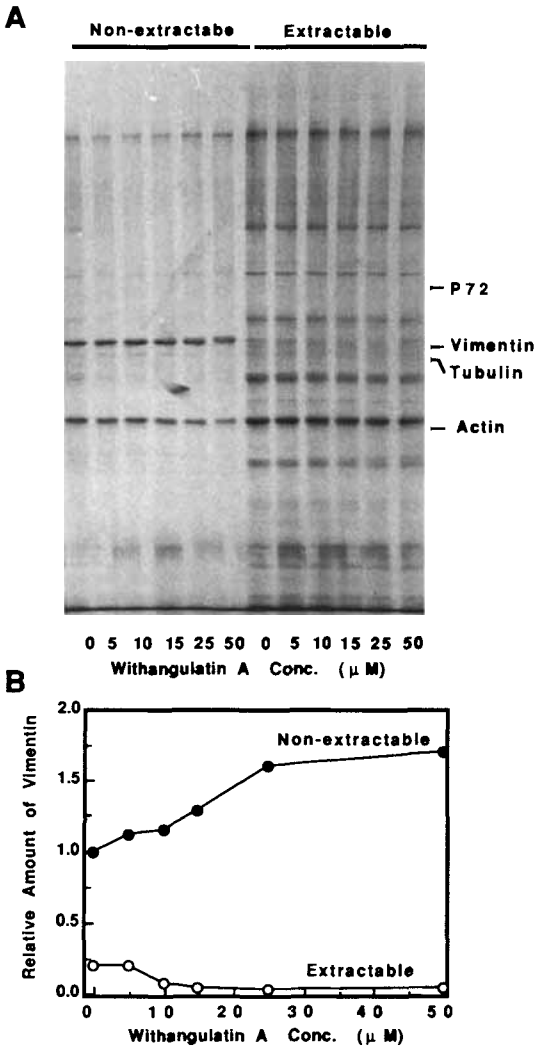

Fig. 6. Concentration-dependent changes in detergent extractability of vimentin induced by withangulatin A in 9L cells. Cells were prelabeled with [^{35}S]methionine for 6 h before they were treated with withangulatin A for 1 h at various concentrations as indicated. After treatments, the cells were lysed with extraction buffer for 30 min and the cellular proteins were collected as extractable and nonextractable fractions. The samples were resolved by SDS-PAGE and the gels were processed for autoradiograph (**A**). The locations of actin, tubulin, and vimentin were indicated on the right. P72: protein with molecular weight of 72,000. (**B**) The autoradiograph shown in (A) was scanned on a laser densitometer. The relative amount of vimentin was calculated from the peak area relative to that of actin in the same lane.

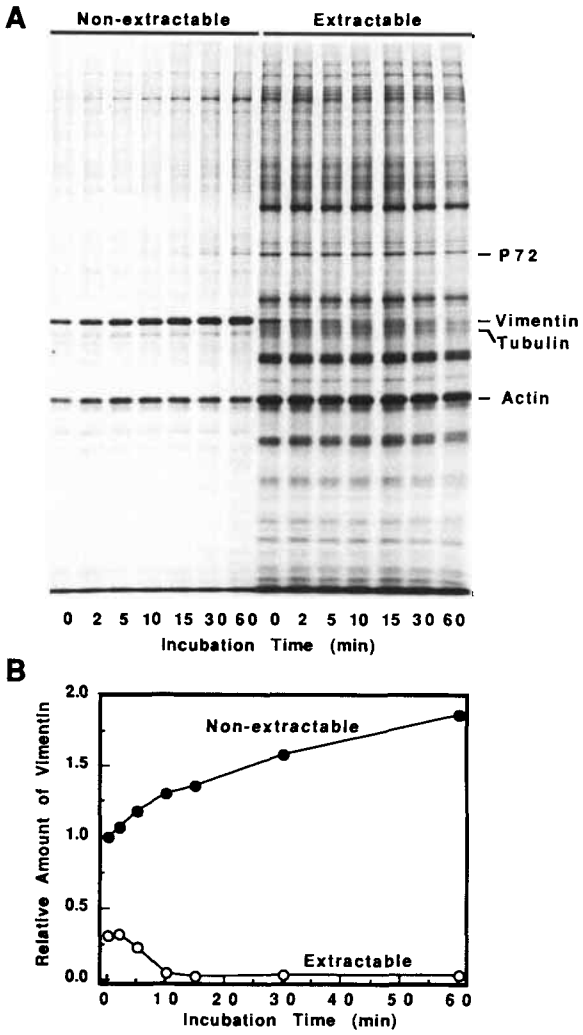

Fig. 7. Time-dependent changes in detergent extractability of vimentin induced by withangulatin A in 9L cells. Cells were pre-labeled with [^{35}S]methionine for 6 h before they were treated with 50 μM of withangulatin A for various durations as indicated. After treatments, the cells were lysed with extraction buffer for 30 min and the cellular proteins were collected as extractable and nonextractable fractions. The samples were resolved by SDS-PAGE and the gels were processed for autoradiography (**A**). The locations of actin, tubulin and vimentin were indicated on the right. P72: protein with molecular weight of 72,000. (**B**) The autoradiograph shown in (A) was scanned on a laser densitometer. The relative amount of vimentin was calculated from the peak area relative to that of actin in the same lane.

Effect of Withangulatin A on the Detergent Extractability of HSP72

In addition to the decrease of extractable vimentin occurred in the withangulatin A-treated cells, decrease in extractability of other proteins was also examined. In the 10 min extraction protocol, the extractability of vimentin by withangulatin A was not as significantly lowered as that of 30 min extraction protocol (compared Figs. 6A and 7A to Fig. 8A,B). However, decrease in extractability of other proteins became more obvious. The proteins associated with modified vimentin in the nonextractable fraction

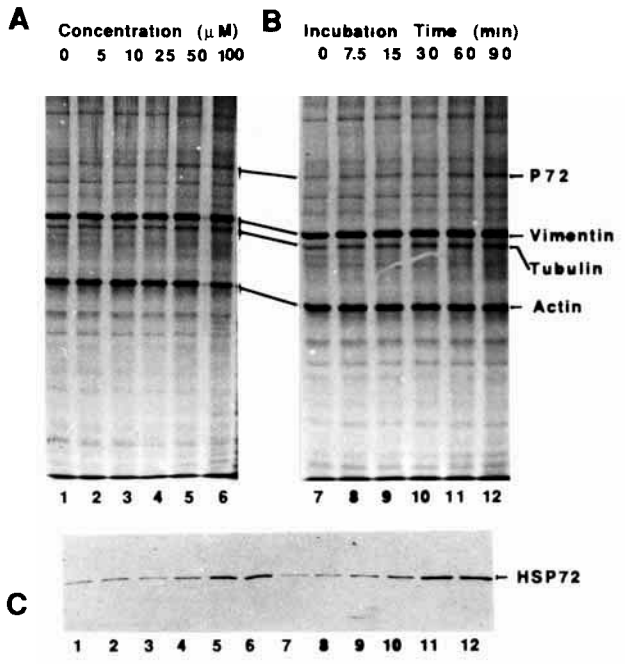

Fig. 8. Effect of withangulatin A on the association of vimentin and HSP72 in the detergent nonextractable fractions in 9L cells Cells were prelabeled with [^{35}S]methionine for 6 h before they were incubated with various concentrations of withangulatin A for 1 h or 50 μM withangulatin A for various durations as indicated After treatments, the cells were lysed with extraction buffer for 10 min and the nonextractable fractions were collected The samples were resolved by SDS-PAGE and the gels were processed for autoradiography (**A,B**) and immunoblotting (**C**) by using antibody against HSP72 as a probe HSP72, constitutive heat-shock protein with molecular mass of 72,000 Lanes 1–6, cells treated with 0, 5, 10, 25, 50, and 100 μM withangulatin A for 1 h, respectively, Lanes 7–12, cells treated with 50 μM withangulatin A for 0, 7 5, 15, 30, 60, and 90 min, respectively

included those with molecular weights of 72,000, 90,000, and 110,000 (designated as p72, p90, and p110, respectively). By immunoblot analysis, the identity of p72 was determined to be HSP72 (Fig. 8C). The data also showed that the amount of HSP72 associated with modified vimentin in nonextractable fraction increased when the cells were treated with increased doses of withangulatin A (Fig. 8C). Further analysis indicated that change in the detergent extractability of HSP72 coincided with that of vimentin and that the amounts of these two protein partitioned into the nonextractable fraction under various doses of withangulatin A were well correlated (Fig. 9).

DISCUSSION

We have showed that cells treated with withangulatin A changed to a round-up morphology and this process was accompanied by the reorganization of the cytoskeletal components. Vimentin is a major cellular protein and the only intermediate filament protein in 9L rat brain tumor cells [Lee et al., 1992]. The ability of withangulatin A to disrupt vimentin intermediate filament was evidenced by the unique characteristic changes of vimentin molecules including augmentation of phosphorylation, retardation of electrophoretic mobility, and decrease in detergent extractability. In addition, HSP72 was found to be co-localized with vimentin in the detergent nonextractable fraction in cells treated with withangulatin A.

The reorganization and/or depolymerization of intermediate filaments may be accomplished by one of two possible mechanisms: a change in the degree of phosphorylation or degradation by calcium-activated, intermediate filament-specific protease [Bloemendal and Pieper, 1989]. In the presence of withangulatin A, the level of vimentin increased by 1.2-fold (data not shown), which does not account for a 2.5-fold increase in

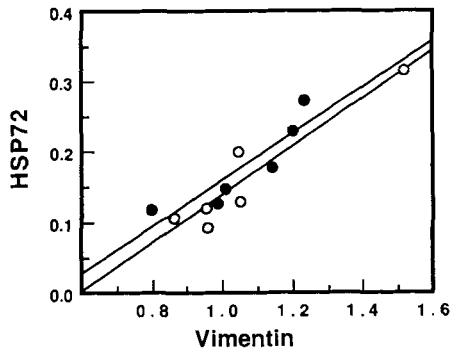

Fig. 9. Correlation of the amounts of HSP72 and vimentin co-localized in the detergent nonextractable fraction in cells treated with different doses of withangulatin A. The relative amounts of proteins were obtained from Figure 8A and B by densitometry. Closed symbols and the upper line represent the time-dependent changes, $r^2 = 0.791$; open symbols and the lower line represent the concentration-dependent changes, $r^2 = 0886$.

the phosphorylation level of this protein. Therefore, an alteration of the protein kinase-phosphatase system may be involved in the process. Increased phosphorylation of vimentin coincides temporally with changes in intermediate filament organization, such as the formation of a "cage" of intermediate filaments surrounding the spindle during mitosis [Zieve et al., 1980]. The entering of mitotic phase is reported to be mediated by the activation of $p34^{cdc2}$ protein kinase which has recently been identified as the catalytic subunit of vimentin kinase [Norbury and Nurse, 1989; Chou et al., 1990] and it has been shown that the phosphorylation of vimentin by the $p34^{cdc2}$ kinase is responsible for the disassembly of the intermediate filaments [Chou et al., 1989]. Increase in phosphorylation of vimentin has been observed in cells subjected to a variety of treatments such as cAMP increasing agents [Gard and Lazarides, 1982; Coca-Prados, 1985; Lamb et al., 1989], chemotactic factors [Huang et al., 1984; Wyatt et al., 1991], phorbol ester PMA [Huang et al., 1988], and, most recently, okadaic acid [Yatsunami et al., 1991; Lee et al., 1992]. Since these effects are not entirely cell cycle dependent, these results suggest that vimentin may be a substrate for a number of protein kinases. On the other hand, dephosphorylation of vimentin seems to be regulated by a Ca^{2+}-stimulated and/or okadaic acid sensitive protein phosphatases, PP1 and PP2A [Evans, 1989; Yatsunami et al., 1991; Lee et al., 1992]. Although it is possible that withangulatin A may directly stimulate/inactivate the respective vimentin kinase/phosphatase, we inferred that withangulatin A-induced conformational changes of vimentin is mediated through the alteration its substrate reactivity with respect to the kinase/phosphatase enzyme system. Further identification of the kinases and phosphatases participating in the phosphorylation-dephosphorylation of vimentin is warranted.

The pattern of characteristic "staircase" of degradation products of vimentin on two-dimensional gels as shown in Figure 5 is identical to that of documented [O'Connor et al., 1981; Spruill et al., 1983; Isaacs et al., 1989]. In contrast, the degradation products of vimentin were not observed in SDS-polyacrylamide gels. This may be due to limited degradation of vimentin, presumably via Ca^{2+}-activated protease [Nelson and Traub, 1982, 1983], during cell harvesting with different buffers. The changes in electrophoretic mobility of vimentin induced by withangulatin A is not likely due to the phosphorylation state of vimentin, since the polypeptide VDP4 (Fig. 3) was neither phosphorylated in control nor in withangulatin A-treated cells but its mobility on two-dimensional gels was heavily retarded (Fig. 5). In addition, the fact that all VDPs were retarded indicates that the modification site may locate in the domains other than the 9 kDa N-terminal headpiece which contains the majority of phosphorylation sites. Preliminary studies indicate that Ca^{2+} binding, ADP ribosylation and ubiquitination were unlikely to be responsible for the changes in electrophoretic mobility of vimentin in withangulatin A-treated cells (data not shown). Therefore, decrease in detergent extractability and retardation of electrophoretic mobility of vimentin may represent changes in protein conformation as noted by Roy et al. [1991]. Each vimentin promoter consists of a central rod-like portion rich in α-helical domains, flanked by amino-terminal and carboxy-terminal nonhelical regions, respectively termed headpiece and tailpiece; both are involved in linking the subunits to form filaments and interprotofibril associations [Steinert and Roop, 1988; Bloemendal and Pieper, 1989]. On the other hand, the rod domain is responsible for subunit association (prior to the fibril formation) by virtue of hydrophobic interactions and it has been reported that cholesterol is able to bind this rod domain [Traub et al., 1985, 1986; Perides et al., 1987]. Withangulatin A is a steroidal lactone containing a sterol backbone which

is closely related to the structure of cholesterol [Chen et al., 1990]. Therefore, it is conceivable that this compound exerts its effect by direct binding to vimentin filaments and such binding may disturb the conformation of the vimentin molecules which is reflected in the changes of detergent extractability and electrophoretic mobility. In addition, the binding of withangulatin A to vimentin filaments may simultaneously disrupt its filamentous organization.

Our previous findings demonstrated that withangulatin A can induce a typical stress response in cells including inhibition of general protein synthesis and induction of heat-shock proteins [Lee et al., 1991]. We have now determined that vimentin modifications was augmented in the cells treated with withangulatin A and the process was dose dependent. Moreover, vimentin modifications appear to be enhanced by withangulatin A within the dose ranges at which the suppression of general protein synthesis and induction of HSPs is also detected [Lee et al., 1991]. Our observations concur with the notions that protein synthesis is affected by the integrity of intermediate filaments [Shyy et al., 1989] and that disruption of cytoskeleton may play a role of signalling for stress response [Biessmann et al., 1982; Thomas et al., 1982; Welch and Suhan, 1985; Shyy et al., 1989]. On the other hand, there are several lines of evidence indicate that abnormal or denatured proteins may be responsible for the induction of HSPs synthesis [Hightower, 1980; Hightower and White, 1981; Ananthan et al., 1986; Lee and Hahn, 1988; Edington et al., 1989]. This proteotoxic hypothesis has become one of the leading concepts in explaining the mechanism underlying the heat-shock response, especially the induction of HSPs [Hightower, 1991]. Since withangulatin A mimics the heat-shock response in 9L cells, it is conceivable that treatment of withangulatin A may cause protein denaturation and that the modified form of vimentin resulted from the treatment of withangulatin A may represent a type of denatured protein. HSP72 has also been shown to be associated with a number of cellular proteins (presumably denatured) during or after stress treatment [Collier and Schlesinger, 1986; Napolitano, et al., 1987; Dubois et al., 1989]. Thus, it is conceivable that the level of homeostatic HSP72 is depleted after stress treatment [Schlesinger et al., 1990]. If the affinity of denatured proteins with HSP72 is higher than that of normally complexed proteins, HSP72 would dissociate from protein complexes during stress and bind to the denatured proteins generated from stress. The dissociation of protein complexes itself might play an essential role for sensing and signalling stress response, including heat-shock gene regulation [Abravaya et al., 1992; Baler et al., 1992; Beckmann et al., 1992]. In fact, it has recently been demonstrated that HSP72 is bound to the heat shock transcription factor in an inactive complex and the dissociation of HSF from this complex results in the activation of HSF which transactives the heat-shock genes [Abravaya et al., 1992]. In the present study, HSP72 is coinsolubilized with proteins including vimentin in the withangulatin A treated and heat-shocked cells (Figs. 8, 9). Because vimentin is abundant in 9L cells, it is possible that vimentin is the major denatured protein to be chaperoned by HSP72 after stress treatment and the process leads to the activation of HSF and initiation of stress response.

In summary, we have demonstrated that withangulatin A can induce modifications of vimentin which leads to a round-up morphology of, and vimentin-HSP72 association in, 9L cells and that the association of HSP72 with modified vimentin may be responsible for the induction of heat-shock response. Moreover, it should be noted that withangulatin A may be used as a valuable tool to study the mechanisms underlying assembly-disassembly and elucidate the function(s) of intermediate filaments in living cells.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council of R.O.C. (NSC 81-0211-B-007-04 and NSC 82-0211-B-007-004) to Y.-K. Lai. The authors wish to thank Dr. S.-D. Yang and Dr. J.-L. Yang for their valuable discussions.

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