A heat-shock-like response with cytoskeletal disruption occurs following hydrostatic pressure in MG-63 osteosarcoma cells

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HASKIN, C.L., ATHANASIOU, K.A., KLEBE, R., and CAMERON, I.L. 1993. A heat-shock-like response with cytoskeletal disruption occurs following hydrostatic pressure in MG-63 osteosarcoma cells. Biochem. Cell Biol. 71: 361–371.

Human osteosarcoma cells, MG-63, were exposed to a hydrostatic pressure shock of 4.0 MPa for 20 min. Changes in subcellular distribution of the cytoskeletal elements and heat shock protein 70 (hsp70) were followed by indirect immunofluorescence and by avidin–biotin–peroxidase protocols. During recovery, total cellular RNA was determined and actin and aldolase mRNA content was followed using reverse transcription – polymerase chain reaction techniques. Hydrostatic pressure caused cell rounding (but not cell death), disruption of microtubules, collapse of intermediate filaments to a perinuclear location, collapse of actin stress fibers into globular aggregates in the cytoplasm, and the formation of several large elongated intranuclear actin inclusions. During recovery, the cells flattened, reorganized microtubules, and redistributed intermediate filaments prior to the reappearance of actin stress fibers. At 20 and 60 min following the initiation of hydrostatic pressure, there was increased anti-hsp70 staining at the nuclear membrane and concentration of hsp70 in four to six granules in the nucleus. At 120 min following the hydrostatic pressure, hsp70 showed intense staining in the cytoplasm and hsp70-containing granules in the nucleus disappeared. Cellular RNA decreased during the first 120-min posthydrostatic pressure shock and then recovered to near prehydrostatic pressure treatment levels by 240 min. Actin mRNA abundance, in relation to aldolase mRNA abundance, showed the same temporal pattern of initial decrease, followed by increase as did total RNA. Review of the literature indicated that eukaryotic cells respond to heat shock and to hydrostatic pressure by disruption of the cytoskeletal elements and by similar modifications in genetic expression. In this study, the observed responses of MG-63 cells to a 4-MPa hydrostatic pressure shock was like the reported response of mammalian cells to a 43°C heat shock.

Key words: heat shock response, hydrostatic pressure, cytoskeleton.

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Des cellules MG-63 d'ostéosarcome humain ont été soumises à une pression hydrostatique de 4,0 MPa pendant 20 min. Les modifications de la distribution cellulaire des éléments du cytosquelette et de la protéine du choc thermique hsp70 ont été suivies par immunofluorescence indirecte et par la technique de l'avidine–biotine-peroxydase. Pendant la période de récupération, l'ARN cellulaire total a été mesuré et les quantités des ARNm de l'actine et de l'aldolase ont été déterminées par des techniques de transcription inverse en chaîne. La pression hydrostatique entraîne un arrondissement des cellules (mais non leur mort), une dépolymérisation des microtubules, une accumulation des filaments intermédiaires autour du noyau, une contraction des fibres de stress d'actine qui forment alors des agrégats globulaires cytoplasmiques et la formation de plusieurs grosses inclusions d'actine allongées intranucléaires. Pendant la récupération, les cellules s'aplatissent, les microtubules se réorganisent et les filaments intermédiaires se redistribuent avant la réapparition des fibres de stress d'actine. Vingt et soixante minutes après le début de l'exposition à la pression hydrostatique, le marquage avec un anticorps anti-hsp70 augmente près de la membrane nucléaire et l'hsp70 est concentrée dans quatre à six granules intranucléaires. Puis, 120 min après l'exposition à la pression hydrostatique, il y a un marquage très prononcé de l'hsp70 dans le cytoplasme et les granules intranucléaires contenant l'hsp70 disparaissent. Le taux d'ARN diminue dans les cellules durant les premières 120 min après le choc de pression hydrostatique, puis il augmente de nouveau et atteint presque le taux antérieur à l'exposition à la pression hydrostatique après 240 min. La quantité de l'ARNm de l'actine, relativement à celle de l'ARNm de l'aldolase, varie en fonction du temps comme le taux d'ARN total : elle diminue initialement puis elle augmente. Une revue de la littérature indique que les cellules eucaryotes répondent à un choc thermique et à la pression hydrostatique par une dépolymérisation des éléments du cytosquelette et par des modifications similaires de l'expression génique. La réponse des cellules MG-63 à l'exposition à une pression hydrostatique de 4 MPa, observée dans cette étude, est similaire à la réponse des cellules de mammifère à un choc thermique de 43°C décrite dans la littérature.

Mots clés : réponse à un choc thermique, pression hydrostatique, cytosquelette.

[Traduit par la rédaction]

Introduction

Ritossa (1962) first observed a heat-induced alteration in the pattern of chromosome puffs in the giant salivary gland cells of *Drosophila*. Exposure to supraoptimal temperatures resulted in the loss of most of the existing puffs and the rapid appearance of seven to nine new puffs (Ashburner and Bonner 1979). The induced expression of a special set of heat-response proteins in *Drosophila* was first linked to Ritossa's observations by Tissieres et al. (1974). Heat shock proteins have subsequently been found to be induced in cells of all types tested when stressed by supraoptimal temperatures or various other stressors. It is now known that there are multiple heat shock protein families (Southgate et al. 1985). Heat shock proteins with a mass of about 70 kDa constitute a family of highly conserved proteins. Some hsp70 proteins are expressed constitutively, while others show inducible gene expression, in response to various stress conditions including heat shock, heavy metals (Welch 1990), oncogene and protooncogene activation, fever, inflammation, neuronal injury, aging (Morimoto et al. 1992), and increased hydrostatic pressure (Haskin et al. 1993). A major role of hsp70 is to bind to and refold partially denatured or misfolded proteins, and to help disassociate abnormal protein aggregates. In addition, hsp70 plays a role as a chaperone that linearizes proteins for translocation across membranes such as endoplasmic reticulum, mitochondria, or other organelles (Langer and Neupert 1991), and for protein import into the nucleus (Dingwall and Laskey 1992; Shi and Thomas 1992). Hsp70 also appears to aid in the assembly of microtubules (Gupta 1990).

The heat shock response includes (*i*) cell cycle arrest (Rao and Engelberg 1965; Zeuthen 1971); (*ii*) decreases in intracellular pH and ATP levels (Leenders et al. 1974; Findly et al. 1983; Weitzel et al. 1985; Drummond et al. 1986); (*iii*) disruption of RNA processing (Muhich et al. 1989; Yost et al. 1990; Welch 1990); (*iv*) increased perichromatin granules due to accumulation of aggregates of unprocessed RNA (Heine et al. 1971; Mayrand and Pederson 1983; Yost and Lindquist 1986; Welch and Mizzen 1988; Welch 1990 and references therein); (*v*) a reduction in the synthesis of large ribosomal RNAs and 5S RNAs, a process dependent on continuous synthesis of ribosomal proteins (Simard and Bernhard 1967; Ellgaard and Clever 1971; Rubin and Hogness 1975; Yost et al. 1990); (*vi*) selective degradation of mRNAs (Lindquist 1980); (*vii*) suppression of normal protein synthesis (Yost et al. 1990 and references therein); (*viii*) the appearance of actin-containing inclusions within the nucleus (Pekkala et al. 1984; Welch and Suhan 1985; Iida et al. 1986); (*ix*) loss of actin filament organization in the cytoplasm (Glass et al. 1985; van Bergen en Henegouwen et al. 1985; van Bergen en Henegouwen and Linnemans 1987; Shyy et al. 1989; Kitano and Okada 1990); (*x*) microtubule disassembly (van Bergen en Henegouwen et al. 1985; van Bergen en Henegouwen and Linnemans 1987; Kitano and Okada, 1990); (*xi*) collapse of intermediate filaments into a perinuclear network (Shyy et al. 1989; Welch 1990); and (*xii*) relocalization of organelles (Welch and Suhan 1985).

Several responses similar to the heat shock response have also been reported following exposure of cells to hydrostatic pressures in the 2–20 MPa range, including (*i*) cell cycle arrest and decreased DNA synthesis; (*ii*) disruption of polysomes (Marsland 1970; Murakami 1970; Zimmerman and Zimmerman 1970; Reed et al. 1993); (*iii*) disruption of RNA synthesis and processing (Landau 1970; Zimmerman et al. 1987); (*iv*) suppression of normal protein synthesis (Landau 1970; Zimmerman et al. 1987); (*v*) disassembly of cytoskeletal components, inhibition of cytokinetic activity, and cell rounding (Marsland 1970; Zimmerman and Zimmerman 1970; Swezey and Somero 1985; Otter et al. 1987; Bourns et al. 1988; Haskin et al. 1993); (*vi*) loss of actin filament organization in the cytoplasm (Somero 1992; Haskin et al. 1993); (*vii*) microtubule disassembly (Zimmerman and Zimmerman 1970; Haskin et al. 1993); (*viii*) collapse of intermediate filaments into a perinuclear network (Haskin et al. 1993); and (*ix*) relocalization of organelles (Zimmerman and Zimmerman 1970).

We report further evidence that hydrostatic pressure induces a heat shock type response in MG-63 osteosarcoma cells and that this response appears to be linked to the state of cytoskeletal disassembly. Briefly, cells that exhibited a greater cytoskeletal disassembly demonstrated a more pronounced heat shock response than cells that underwent a minimal loss of cytoskeletal organization. That both hydrostatic pressure and heat shock are shown to result in a parallel sequence of cytoskeletal and hsp70 responses suggests that the cytoskeletal elements, particularly actin, play an essential role in the cell's transcriptional regulation of hsp70 genes when exposed to environmental stressors.

Methods

Tissue culture

MG-63 cells (a human osteosarcoma cell line obtained from American Type Culture) were maintained in Dulbecco's modified essential media (Sigma, D-1152) supplemented with 10% fetal bovine serum (Sigma, F-4010) and glutamine (Sigma, G-7513). Cells to be used in immunohistochemistry and immunofluorescence studies were seeded onto uncoated masked glass slides (Carlson Scientific, 100808), cultured for 24 h in a high humidity chamber with 5% CO_2 at 37°C, and then treated with a single 20-min hydrostatic pressure pulse of 4.0 MPa. Cell culture slides were fixed in ice-cold acetone for 5 min immediately following the pressure pulse and at 15, 30, 45, 60, and 120 min after the pressure pulse. Fixed slides were stored at -20°C until the immunofluorescence assay. MG-63 cells (1×10^6 cells/60-mm plate) used for RNA isolation were grown on uncoated glass Petri dishes and treated with a single 20-min pressure pulse of 4.0 MPa. Total RNA was isolated from control and hydrostatic pressure treated cultures using the single-step protocol of Chomczynski and Sacchi (1987), as modified and described below.

Pressure treatments

Prior to the selected pressure treatment, MG-63 cells were incubated in a high humidity chamber with 5% CO_2 at 37°C and then transferred to the pressure cytochamber as previously described (Haskin et al. 1993). This closed-loop, computer-controlled cytochamber system allows accurate pressurization without oscillations at constant temperature (Athanasiou et al. 1993). A single 4.0-MPa pressure pulse of 20 min of duration was used for both the RNA and the immunohistochemistry studies. Experimental controls included cell cultures maintained in the high humidity incubator and cell cultures treated at ambient atmospheric pressure in the cytochamber for the same length of time as the experimental groups. The pressure pulse curves were symmetrical, i.e., the rate at which the pressure was increased was equal to the rate at which the pressure was released at the end of the 20-min pulse. The variation in temperature during the 20-min pulse was ± 1°C. Because the process of transferring cell cultures to the cytochamber briefly exposes the cultures to the air, antibiotics

TABLE 1. Changes in area and morphology of MG-63 osteosarcoma cells in response to hydrostatic pressure

Time after HP (min)	Average area (μm^2) (n)	% rounded (length = width)	% partially rounded (length > width)	% flattened (length >>> width)
20	0.1812 ± 0.05* (72)	37	40	23
80	0.2192 ± 0.07 (75)	27	48	25
140	0.2402 ± 0.07 (73)	13	40	47
Control	0.4573 ± 0.13 (68)	05	10	85

*± standard deviation.

(penicillin–streptomycin, Sigma P-0781) were added to the media following the pressure pulse.

Indirect immunofluorescence

A panel of antibodies was selected to demonstrate changes in cytoskeletal organization in response to physiological levels of pressure. A polyclonal anti-actin antibody (Sigma, A2668) that recognizes multiple epitopes was used to assay actin organization so that different forms of actin, including f-actin and g-actin, could be visualized. Additional antibodies, including monoclonal antibodies against α-tubulin (Sigma, T9026), β-tubulin (Sigma, T0426), and vimentin (Oncogene Science, 1F01), were also used to evaluate the cytoskeletal organization. Anti-hsp (Oncogene Science, HSP01) monoclonal antibody was used to evaluate the stress response to HP. Fixed slides stored at −20°C were rehydrated, blocked using 5% BSA in Dulbecco's PBS (Sigma, D-7030), and incubated for 45–60 min at room temperature with one of the antibodies. Preparations were rinsed three times in PBS–BSA, blocked with 1% goat serum (Sigma, S-6898), and then incubated with FITC-labeled secondary anti-IgG antibodies (Sigma, F5387 or F0511) for 30 min at 4°C. Preparations were counterstained with propidium iodide, as needed, to locate nuclei, rinsed three times in PBS–BSA, and prepared for microscopic evaluation. Photomicrographs were taken using a Zeiss fluorescent photomicroscope using ASA 1600 35-mm film.

Immunohistochemistry and morphometry

Localization of hsp70 was also assayed by an avidin – biotin – horseradish peroxidase and DAB protocol as described by Stewart et al. (1986). Briefly, slides were fixed in ice-cold acetone for 5 min, air dried, rehydrated with PBS–BSA, treated with H_2O_2 to inactivate endogenous peroxidase activity, incubated with the anti-hsp70 monoclonal antibody, rinsed in PBS–BSA, incubated with biotinylated anti-IgG, rinsed, treated with avidin – biotin – horseradish peroxidase complex, rinsed, incubated with DAB substrate, and washed. Photomicrographs were taken using a Zeiss photomicroscope using Kodak high-contrast copy film.

Morphometric measurements were carried out using a digitizing table and the Bioquant II image analysis system (R & M Biometrics, Nashville, Tenn.) on a Leitz Diaplan microscope. Measurements consisted of (i) area seen by transillumination of the stained cells; (ii) maximum length of cell drawn through the cell nucleus, and (iii) maximum width of cell drawn through the cell nucleus.

RNA isolation and polymerase chain reaction

Total RNA was isolated from 1×10^6 cells from control and hydrostatic pressure treated cultures using the single-step protocol of Chomczynski and Sacchi (1987), quantitated by OD at 260 and 280 nm. The relative abundance of actin and aldolase mRNAs was assayed by reverse transcription – polymerase chain reaction techniques. Primer sequences of the desired size were selected based upon their lack of homology with other known DNA sequences,

low GC content (35%), and appropriate melting points. PCR primers were synthesized by the Biopolymer Sequencing and Synthesis Facility in the Department of Biochemistry (University of Texas Health Science Center at San Antonio, Tex.) on a Cyclone DNA synthesizer (Milligen/Biosearch, Burlington, Mass.) and purified by OPC cartridge. One microgram of total RNA isolated from each condition was used in single-tube reverse transcription reactions to obtain cDNAs for use in PCR amplification.

The one-tube RT–PCR method used (Conboy et al. 1988) was modified to yield free MgCl₂ (rather than dNTP bound), 2 mM Taq DNA polymerase, and 6 mM avian myeloblastosis virus reverse transcriptase. Actin and aldolase cDNAs were generated by using specific oligonucleotide primers to prime reverse transcription in a 20-μL reaction solution as described below. RNA and primer annealing was done in DEPC-treated H_2O at 68°C for 15 min, followed by cooling on wet ice. Following annealing, the reaction solution (40 mM KCl, 50 mM Tris–HCl (pH 8.3), 8 mM MgCl₂, 0.5 mM of each dNTP for a total of 2 mM dNTPs (Pharmacia, Piscataway, N.J.), 200 ng of antisense primer, 10 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.), 20 U of RNAsin (Promega, Madison, Wis.), 5 mM dithiothreitol, and 5 μg BSA) was added to a final volume of 20 μL and the reaction was then incubated at 42°C for 1 h.

Following reverse transcription, PCRs were carried out as follows. The 20 μL reverse transcription reaction solution was diluted with 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl₂, 0.1% gelatin; United States Biochemicals, Cleveland, Ohio) to a final volume of 100 μL and a final MgCl₂ concentration of 3 mM (2 mM free MgCl₂), with 100 ng of ³²P-5′-end labeled sense oligonucleotide primer, 100 ng of cold sense primer, additional dNTPs to a concentration of 0.2 mM, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Va.). Amplification was performed on an Ericomp thermocycler (San Diego, Calif.). The reaction mixture was initially denatured at 94°C for 1.5 min and then annealed at 54°C for 1 min, extended at 72°C for 1 min 50 s, and denatured at 94°C for 20 s. The chain reaction was terminated with a final cycle of annealing at 54°C to 56°C for 1 min, and extension at 72°C for 7 min. The resulting DNA fragments were analyzed by electrophoresis on 5% polyacrylamide gels and quantitated by scintillation counting of excised bands.

Results

Immunohistochemistry and immunofluorescence

Hydrostatic pressure caused loss of cytoskeletal organization and resulted in increased cell rounding and decreased cell area (Table 1). Indirect immunofluorescence staining with monoclonal antibodies to actin, vimentin, and α-tubulin demonstrated loss of organization of cytoskeletal proteins in response to 4.0-MPa HP. Because the polyclonal
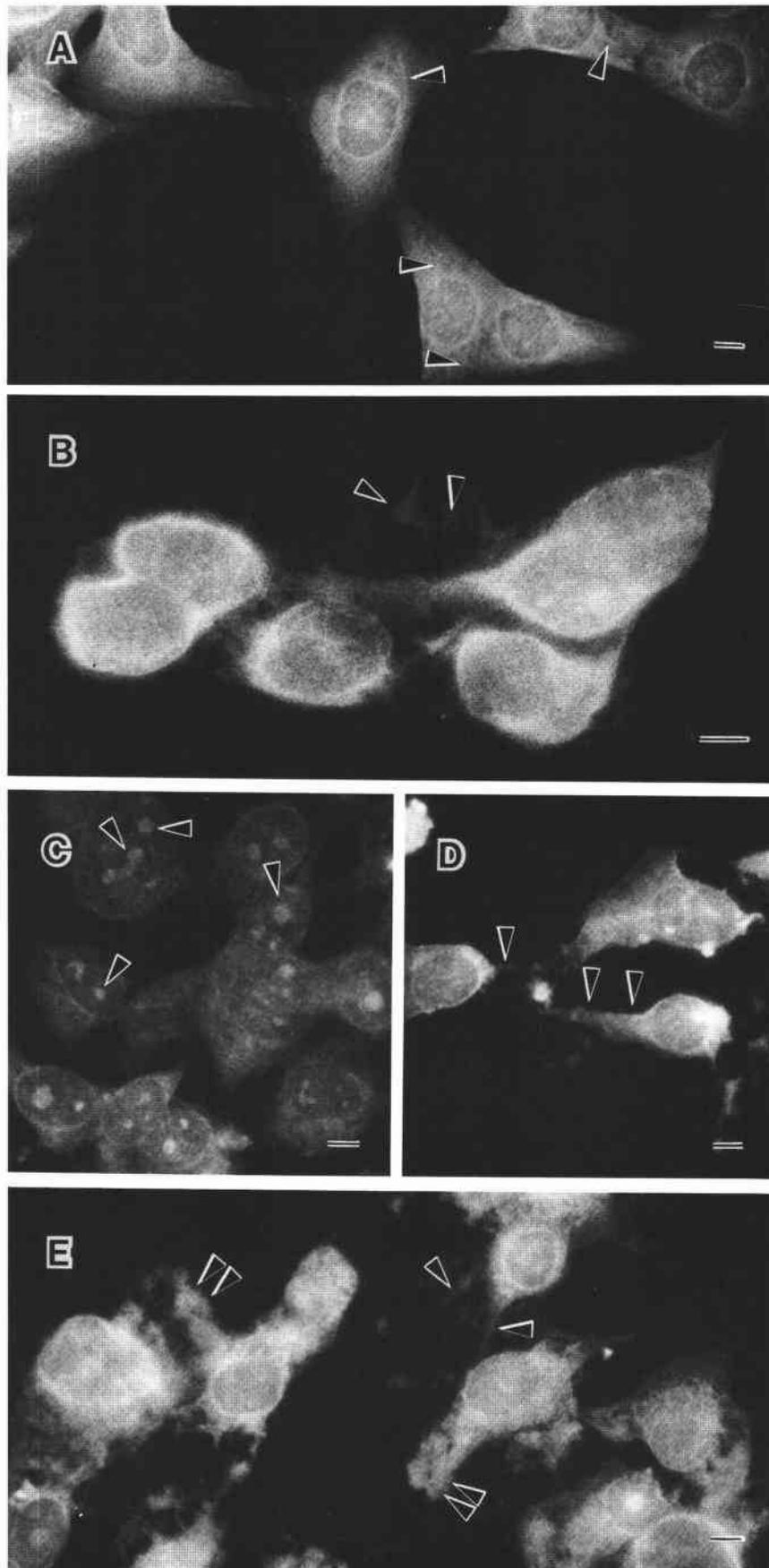
Fig. 1. Indirect immunofluorescence analysis demonstrates time-dependent changes in the distribution of actin stress filaments in MG-63 osteosarcoma cells following a 20-min, 4-MPa HP pulse. MG-63 cells were fixed, processed, and stained using a polyclonal anti-actin antibody and a FITC-conjugated secondary as described in Methods. Each scale bar is 10 μm. (a) Control cultures kept at

anti-actin antibody recognizes multiple actin epitopes, it was possible to visualize actin in different forms, including both f-actin and g-actin and randomly aggregated actin. However, stress fibers were not as apparent as with other staining techniques such as rhodamine-labeled phalloidin because they are partially obscured by the binding of the polyclonal anti-actin antibody with actin in other forms. In nonpressure treated cells, immunofluorescence staining for actin revealed stress fibers that extended throughout the cytoplasm. Immediately following HP, much of the actin staining redistributed towards the nucleus leaving only a few granular concentrations of actin in the cytoplasm (Fig. 1). At 60 min after the hydrostatic pressure pulse, short actin stress filaments began to appear around the nucleus (Fig. 1C). The actin in the cell nuclei was concentrated into two to three large actin granules and the actin in the cytoplasm was present in large aggregates. After 2 h of recovery, most cells had only a few short actin stress fibers around the nucleus, but still had multiple large actin aggregates present in the cytoplasm. Thus, the filamentous cytoskeletal actin organization seen prior to HP treatment did not return to its original organization within the 2-h recovery period. At 2 h, the granular staining pattern in the nuclei was not as evident, although about half of the cells still showed evidence of actin inclusions in the nucleus. Vimentin and α-tubulin also underwent disassembly (see Haskin et al. 1983), collapsing to a perinuclear area, but, unlike actin, did not show staining in the nucleus, and both vimentin and tubulin underwent significant reorganization, towards that seen in the untreated control cells, within the 2-h recovery period.

HP caused translocation of hsp70 from the cytoplasm to the nucleus. This translocation was typical of a heat shock or stress response. There was a graded hsp70 translocation response between different cells in the same exposure chamber, such that the staining intensity of the anti-hsp70 monoclonal within the nucleus was greater in cells that underwent rounding in response to the pressure pulse. At 20 min after the start of HP most of the cells had "rounded up" and disassembled the cytoskeleton in response to the pressure; also hsp70, as assayed by both the indirect immunofluorescence (Fig. 2) and the avidin – biotin – horseradish peroxidase – DAB protocol (Fig. 3), showed nuclear localization. Using indirect immunofluorescence staining, hsp70 was clearly localized to three to six brightly staining granules in the nucleus and to a less brightly stained area around the nuclear membrane. Although the majority of cells had a rounded-up shape immediately after the HP treatment, approximately one quarter of the cells were still somewhat flattened and in these flatter cells the anti-hsp70 monoclonal showed only light perinuclear staining (Fig. 3). After 1-h recovery time following the 20-min pressure treatment, the majority of cells were beginning to regain cytoskeletal organization and hsp70 was concentrated in a nuclear and perinuclear area, but began to show increased staining throughout the cytoplasm (Fig. 3). In about one quarter of the cells, presumably in those cells that had maintained a flattened appearance, the hsp70-staining intensity

was less intense and was primarily perinuclear (Fig. 3). After 2 h of recovery, the majority of the pressure-treated cells had flattened significantly, the nucleus had relatively light anti-hsp70 staining, and the cytoplasm had very intense staining as demonstrated by the avidin – biotin – horseradish peroxidase – DAB protocol (Fig. 3). Although anti-hsp staining was more intense in cells that had undergone cytoskeletal disassembly, even cells that had remained flattened had more cytoplasmic hsp70 than did control cells.

Analysis of total RNA and actin mRNA abundance

Hydrostatic pressure resulted in a time-dependent decrease in actin mRNA abundance (Fig. 4). Actin mRNA levels were generally three times greater than those of aldolase, the selected reference control mRNA. Curve-fit analysis of the ratios of actin to aldolase mRNA abundance, as assayed by RT-PCR of 1 μg of total RNA over time and illustrated in Fig. 4, indicated that the trend of decrease followed by increase was statistically significant at the $p > 0.01$ level (correlation coefficient $= 0.650$, 22 df).

Discussion

Hydrostatic pressure response is similar to heat shock response

We have demonstrated that temperature-controlled (37°C), pressure-treated MG-63 cells have cellular responses similar to those of the heat shock response and that the kinetics of the cellular response to HP correlates well with the kinetics of the cellular response to heat shock stress (Table 2). During and following the hydrostatic pressure pulse, hsp70 concentrated at the nuclear membrane and became localized in the nucleus. Following nuclear localization of hsp70, there was increased staining intensity of hsp70 in the nucleus and the cytoplasm, indicating activation of hsp70 gene expression. During recovery from pressure stress, hsp70 translocated to the cytoplasm. MG-63 cells stressed with 4-MPa HP showed marked changes in cytoskeletal organization including disruption of the microtubules, collapse of intermediate filaments to the perinuclear area (Haskin et al. 1993), loss of actin filament organization and formation of cytoplasmic actin aggregates, and formation of actin inclusion bodies in the nucleus. The increases in cytoplasmic hsp70 during recovery, as assayed by the staining intensity of the anti-hsp70 monoclonal antibody, were correlated with the degree of cytoskeletal disassembly and cell rounding. Time-dependent changes in actin mRNA abundance in response to hydrostatic pressure were similar to those reported for tubulin mRNA (Tahir et al. 1988).

Previous investigators have demonstrated that hydrostatic pressure, like heat shock stress, also triggers (i) immediate cell cycle arrest (Zimmerman and Zimmerman 1970), (ii) changes in cytoskeletal organization within 2 min at the ultrastructure level and within 3 min at the light microscope level (Zimmerman and Zimmerman 1970; Cervera 1978), and (iii) partial inhibition of RNA synthesis within 3 min (Landau 1970), decreased mRNA abundance (Tahir et al. 1988), inhibition of RNA processing (Symington et al. 1991),

ambient pressure demonstrating actin microfilaments throughout the cytoplasm and light-staining actin stress fibers (arrowheads); (b) cells immediately following the HP pulse showing extensive cell rounding, collapse of the actin microfilaments to a perinuclear area, and formation of actin aggregates in the cytoplasm (arrowheads); (c) cells following 1 h of recovery, when cells are still rounded, showing two to three brightly staining actin inclusions in the nucleus (arrowheads); (d) cells following 1 h of recovery, when they are still rounded, showing multiple actin aggregates in the cytoplasm but beginning to reorganize microfilaments; (e) cells after 2 h of recovery showing continued reorganization of microfilaments (arrowheads) and increased actin aggregates in the cytoplasm (double arrowheads).
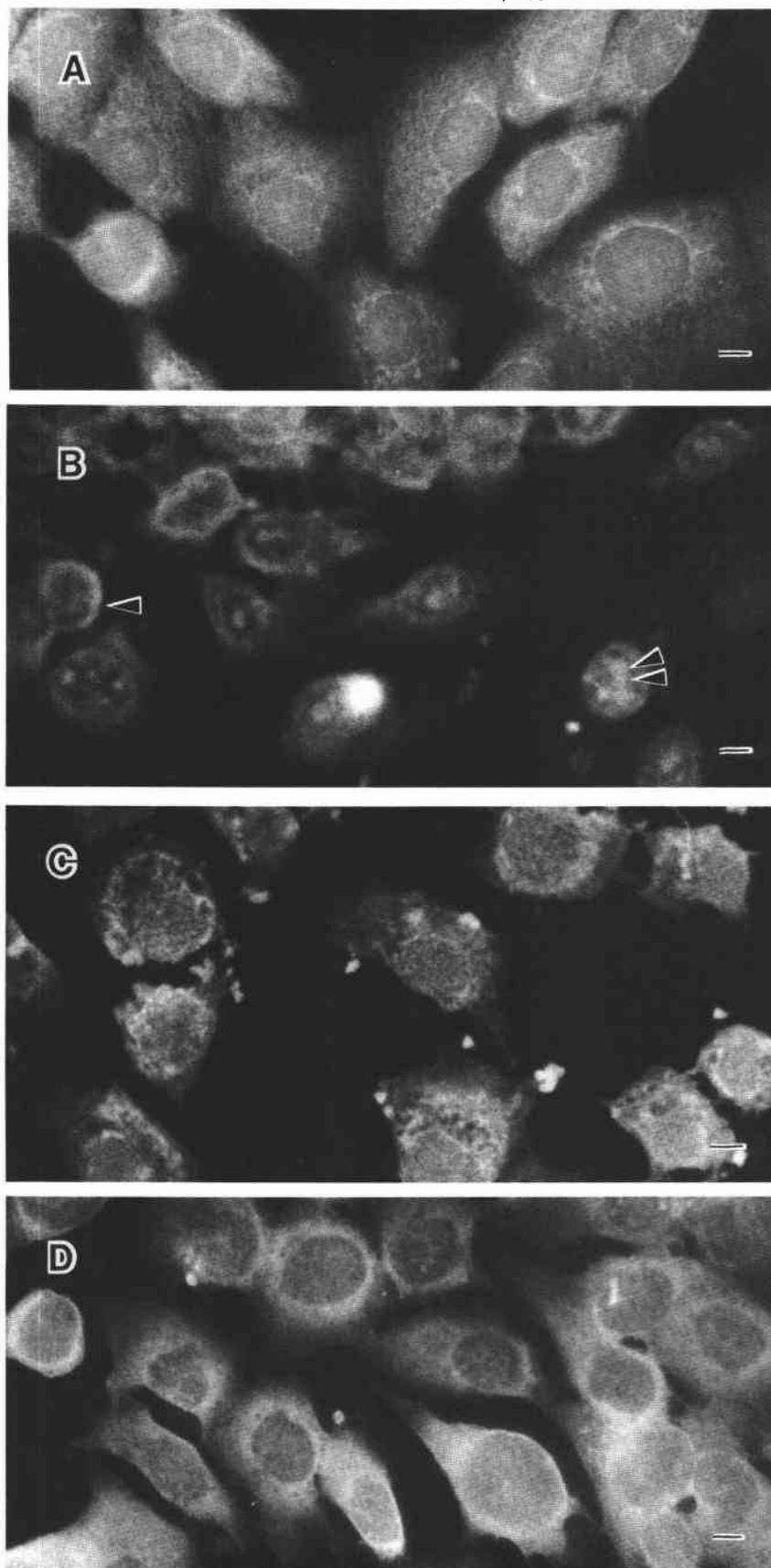
FIG. 2. Indirect immunofluorescence analysis demonstrates time-dependent changes in hsp70 localization following a 20-min, 4-MPa HP pulse. MG-63 cells were fixed, processed, and stained using a monoclonal anti-hsp70 antibody and a FITC-conjugated secondary as described in Methods. Each scale bar is 10 μm. (a) Control cultures kept at ambient pressure demonstrating hsp70 in the cytoplasm and the nucleus; (b) cells immediately following HP pulse showing extensive relocalization of hsp70 to the nucleus (double arrowheads) and perinuclear area (arrowhead); (c) cells following 1 h of recovery showing increased hsp70 levels in the nucleus and cytoplasm; (d) cells following 2 h of recovery showing increased cytoplasmic concentration of hsp70 relative to nuclear hsp70 concentration.
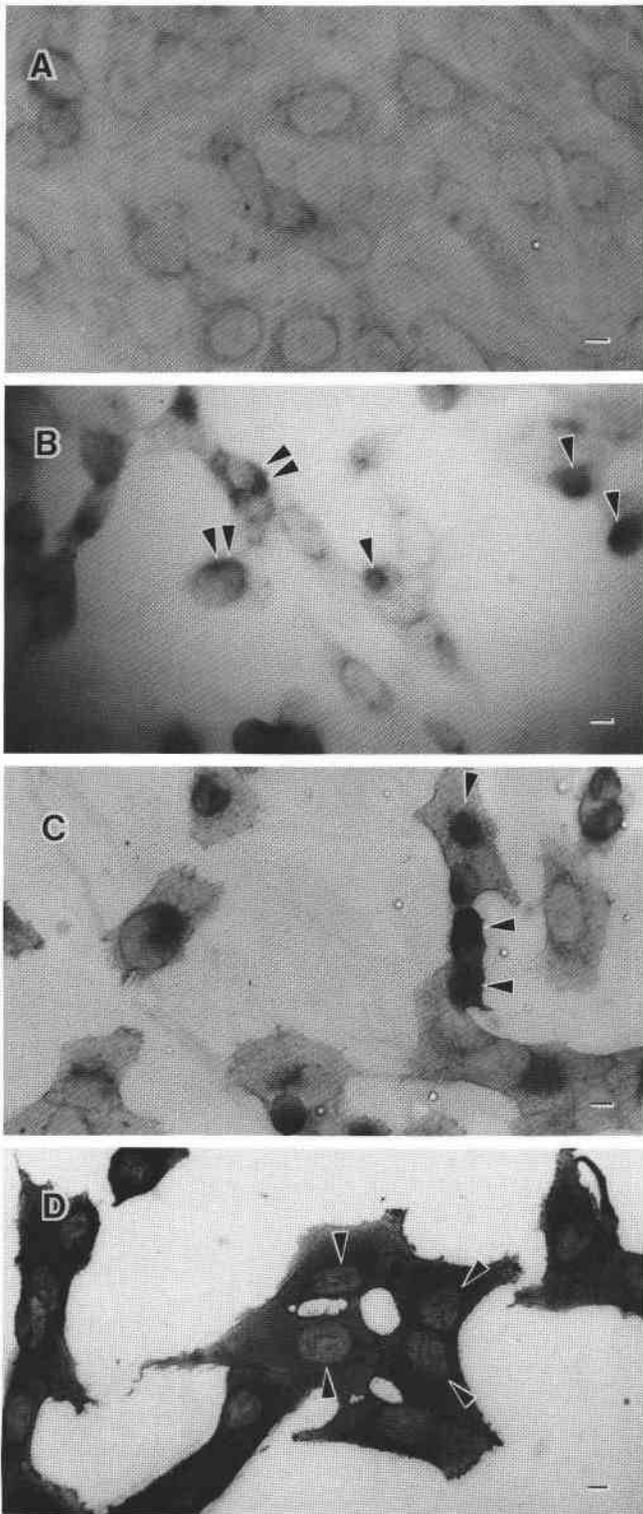
FIG. 3. Avidin – biotin – horseradish peroxidase – DAB analysis demonstrates time-dependent changes in hsp70 localization and concentration of anti-hsp70 following a 20-min, 4-MPa HP pulse. Each scale bar is 10 μm. (*a*) Control cultures kept at ambient pressure demonstrating low levels of hsp70 in the cytoplasm and the nucleus; (*b*) cells immediately following HP pulse showing relocalization and increased concentration of hsp70 in the nucleus (arrowheads) and perinuclear area (double arrowheads); (*c*) cells following 1 h of recovery showing increased hsp70 levels in the nucleus (arrowheads) and cytoplasm (compare with cytoplasmic staining in *a*; (*d*) cells following 2 h of recovery showing redistribution of hsp70 from the nucleus (arrowheads) to the cytoplasm.
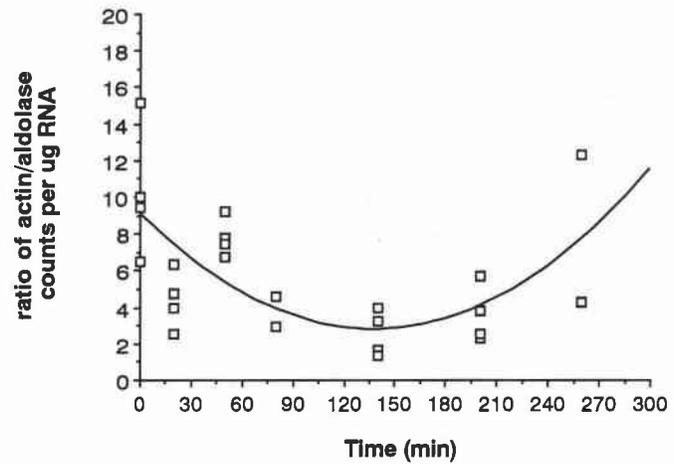

FIG. 4. The abundance of actin and aldolase mRNAs in MG-63 osteosarcoma cells as assayed by RT–PCR techniques as described in Methods. Curve analysis of the actin/aldolase ratios of MG-63 osteosarcoma cells, prior to and following a 20-min, 4-MPa HP pulse indicates that the trend of decrease followed by increase was statistically significant at the $p > 0.01$ level.

and partial inhibition of protein synthesis within 6 min (Landau 1970), followed by resumption of normal protein synthesis within 2–3 h of pressure stress.

The temporal responses to heat shock have been reviewed by Welch (1990). Briefly, normal protein synthesis was inhibited within 3–5 min after a heat shock temperature shift, changes in cytoskeletal organization of mammalian cells were observed within 5–15 min after the temperature shift to 43°C (Pekkala et al. 1984; Glass et al. 1985; van Bergen en Henegouwen et al. 1985; Welch and Suhan 1985; Iida et al. 1986; van Bergen en Henegouwen and Linnemans 1987; Mosser et al. 1988; Shyy et al. 1989; Kitano and Okada 1990), and increased heat shock proteins were detected after 8–10 min (Mirault et al. 1978). Hsp70 is normally found in the cytoplasm at low levels prior to heat shock. After heat shock, hsp70 concentrates at the nuclear membrane, moves into the nucleus, and is either complexed with denatured or aggregated ribosomal proteins (Welch and Suhan 1985; Welch 1990), or with HSF1 and the hsp70 promoter (Morimoto et al. 1992; Morimoto 1993). In addition, there are time-dependent changes in mRNA abundance and disruption of RNA processing (Muhich et al. 1989; Yost et al. 1990). During release from heat shock and as the heat-disrupted nucleoli begin to recover, the hsp70 concentrates in the cytoplasm and is colocalized with translation machinery (Pelham 1984; Velazquez and Lindquist 1984; Welch 1990). In addition to the changes in hsp70 localization, changes in actin and microtubule organization were reported in response to heat shock (Welch and Suhan 1985; Iida et al. 1986; Shyy et al. 1989; Kitano and Okada 1990). Thus, the kinetics of the cellular responses to heat shock and pressure stress are very similar.

The baroprotective effect of heat shock provides additional evidence that the heat shock response and the pressure response are closely related. Although the effect of heat synchronization was not reported as being baroprotective, Zimmerman and Zimmerman (1970) reported that logarithmically growing cells were more sensitive to high pressure than were the heat-synchronized cells and recovery from the effects of pressure was slower in the nonsynchronized cells than in the heat-synchronized cells. This baroprotective

TABLE 2. Sequence of reported events following exposure of eukaryotic cells to hydrostatic pressure or heat shock stress

Time (min)	Hydrostatic pressure	Heat shock
0	Beginning of HP pulse	Beginning of heat shock stress
	Cell cycle arrest (1–3)	Loss of cytoskeletal organization (4, 5)
	Loss of cytoskeletal organization (1, 2, 6–8)	Disruption of organelle location (5, 9)
	Cell rounding (1, 8)	Activation, translocation. DNA binding of HSF1 to HSE (11–15)
	Inhibition of cytokinetic activity (2, 10)	
	Disruption of organelle location (1)	
		Increased hsp70 in nucleus (12)
	Increased hsp70 product in nucleus (12)	Inhibition of transcription of most genes (16)
	Disruption of polysomes (1)	Disruption of RNA processing (16, 17)
	Inhibition of most RNA synthesis (3, 18, 19)	
	Inhibition of RNA processing (20)	Maximum HSF1 levels in nucleus (11, 13)
		Maximum hsp70 transcription (11, 13)
	Inhibition of normal protein synthesis (18, 19)	Increase hsp70 product (11, 13, 21)
		Inhibition of normal cellular protein synthesis (5, 22)
		Decline in HSF1 levels and hsp70 transcription (11)
	Lowest total RNA yield	Hsp70 mRNA remains stable for rest of heat txt (16)
	Increased hsp70 perinuclear	Increased levels of hsp70 in cytoplasm (11)
	Lowest actin mRNA level	Cytoskeletal reorganization begins (4, 5)
	Increased levels of hsp70 in cytoplasm	Resumption of normal protein synthesis
	Cytoskeletal reorganization begins (4, 5, 8)	HSF1 levels at basal (11, 13)
	Actin dots and filaments in cytoplasm (4, 8)	Hsp70 transcription at basal (11)
	Appearance of actin inclusion bodies in nucleus	
		Appearance of actin inclusion bodies in nucleus
260	Total RNA yield at control levels	(9, 23, 24)

NOTES: Numbers in parentheses represent references: 1, Zimmerman and Zimmerman 1970; 2, Marsland 1970; 3, Tahir et al. 1988; 4, Kitano and Okada 1990; 5, Shyy et al. 1989; 6, Bourns et al. 1988; 7, Swezey and Somero 1985; 8, Haskin et al. 1993; 9, Welch and Suhan 1985; 10, Otter et al. 1987; 11, Abravaya et al. 1991; 12, Sarge et al. 1993; 13, Mosser et al. 1988; 14, Morimoto et al. 1992; 15, Morimoto 1993; 16, Yost et al. 1990; 17, Muhich et al. 1989; 18, Landau 1970; 19, Zimmerman et al. 1987; 20, Symington et al. 1991; 21, Craig and Gross 1991; 22, Lindquist 1981; 23, Iida et al. 1986; 24, Pekkala et al. 1984.

effect of heat shock was confirmed and extended by Komatsu et al. (1991), who reported that preincubation of *Saccharomyces cerevisiae* at 40 or 43°C gave significant protection against lethal levels of hydrostatic pressures.

Cytoskeletal organization may be a critical element in adaptation of eukaryotic cells to environmental stress

Many cellular functions that are affected by heat stress or pressure stress depend on the integrity of the cytoskeleton, including (*i*) protein synthesis (Cervera et al. 1981; Ornelles et al. 1986; Hesketh 1991; Vedeler et al. 1991), (*ii*) organelle position (Ball and Singer 1982; Sandoval et al. 1984; Eckert 1986; Matteoni and Kreis 1987), (*iii*) cell shape (Stossel 1984; Luna and Hitt 1992), and (*iv*) mRNA translation and stability (Hesketh 1991; Larson et al. 1991; Vedeler et al. 1991). Additional cellular functions that are affected by heat stress or pressure stress depend on the integrity of the nuclear matrix, including mRNA posttranscriptional processing, transport, translation, and stability (Nakayasu and Ueda 1985; Smith et al. 1986; Zeitlin et al. 1987; Lawrence et al. 1989; Agutter 1991; Smith 1992). Actin, intermediate filament, and nuclear matrix organization are important regulators of RNA processing and transport. The binding of hnRNAs is specific for actin and can be abolished by anti-actin antibodies (Schroeder et al. 1987), and release and export of hnRNAs, but not mature mRNA, occurs in response to depolymerization of actin filaments in the presence of cytochalasin B (Riedel and Fasold 1992 and

references therein). Pre-mRNA, pre-rRNA, and rRNA have also been shown to be associated with the nuclear matrix (Herlan et al. 1979; Smith et al. 1986). Electron microscopy and biochemical studies have demonstrated links between intermediate filaments and the nuclear pores (Franke et al. 1981; Georgatos and Blobel 1987a, 1987b) and that the export of mRNA transcripts occurs along linear tracts at the nuclear pore complexes (Feldherr et al. 1984; Lawrence et al. 1989).

The importance of the cytoskeleton to the heat shock response is also suggested by the evidence that some heat shock proteins are associated with cytoskeletal elements (Reiter and Penman 1983; Leicht et al. 1986; Ohtsuka et al. 1986; Redmond et al. 1989; Tomasovic et al. 1989; Karasev et al. 1992). Although alterations in the cytoskeleton due to heat shock vary from cell type to cell type, some type of cytoskeletal disruption appears to be a common denominator in heat shock response. Reorganization of the cytoskeleton during recovery requires synthesis of new proteins (van Bergen en Henegouwen and Linnemans 1987), resumption of normal protein synthesis is coincident with cytoskeletal reorganization, and prevention of heat shock protein synthesis during stress treatment does not alter the rapid loss of stress fibers (van Bergen en Henegouwen and Linnemans 1987). These observations suggest that cytoskeletal organization may be a critical element in adaptation to environmental stress.

Using indirect immunofluorescence, Sarge et al. (1993)

have shown that prior to heat shock, HSF1, a transcriptional activator for hsp70, is present in low levels in both the cytoplasm and the nucleus of HeLa cells, and that following heat shock, HSF1 has a nuclear localization. Sarge et al. (1993) suggested that HSF1 activation was normally repressed by a titratable negative regulatory factor and demonstrated that recombinant eukaryotic HSF1 expressed in *Escherichia coli* constitutively bound DNA. Since *E. coli* do not have a cytoskeleton, the titratable negative factor may be a binding site on the cytoskeleton that stabilizes the HSF1 monomers and prevents activation, unless HSF1 undergoes a conformational change and (or) release from cytoskeletal elements in response to stress.

We propose that the stress response following HP or heat shock is related to the disruption of cytoskeletal elements. The depolymerization of the cytoskeletal elements may sustain the heat shock response by several possible mechanisms, including (*i*) increased release of HSF1 from depolymerized cytoskeletal components; (*ii*) increased substrates, such as disassembled tubulin or actin, that temporarily deplete the free pool of hsp70; and (*iii*) disassembly of actin in the nuclear matrix in such a way that RNA splicing is disrupted, requiring nuclear localization of hsp70 to repair the damage.

Evidence already exists that supports the role of tubulin dimers as a pool of substrates (Gupta 1990) that depletes cytoplasmic hsp70 pools, and substantial evidence supports the proposal that actin in the nuclear matrix stabilizes RNA processing. However, additional research is needed to determine if HSF1 binds to actin or other cytoskeletal components.

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