High Hydrostatic Pressure Effects In Vivo: Changes in Cell Morphology, Microtubule Assembly, and Actin Organization

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We present the first study of the changes in the assembly and organization of actin filaments and microtubules that occur in epithelial cells subjected to the hydrostatic pressures of the deep sea. Interphase BSC-1 epithelial cells were pressurized at physiological temperature and fixed while under pressure. Changes in cell morphology and cytoskeletal organization were followed over a range of pressures from 1 to 610 atm. At atmospheric pressure, cells were flat and well attached. Exposure of cells to pressures of 290 atm or greater caused cell rounding and retraction from the substrate. This response became more pronounced with increased pressure, but the degree of response varied within the cell population in the pressure range of 290–400 atm. Microtubule assembly was not noticeably affected by pressures up to 290 atm, but by 320 atm, few microtubules remained. Most actin stress fibers completely disappeared by 290 atm. High pressure did not simply induce the overall depolymerization of actin filaments for, concurrent with cell rounding, the number of visible microvilli present on the cell surface increased dramatically. These effects of high pressure were reversible. Cells re-established their typical morphology, microtubule arrays appeared normal, and stress fibers reformed after approximately 1 hour at atmospheric pressure. High pressure may disrupt the normal assembly of microtubules and actin filaments by affecting the cellular regulatory mechanisms that control cytological changes during the transition from interphase into mitosis.

Key words: stress fiber, cytoskeleton, microvilli, tubulin

INTRODUCTION

Subjecting surface dwelling organisms to the hydrostatic pressures of the deep sea (200–1,000 atm) induces significant changes in cell morphology and activities known to be dependent upon the actin or microtubule components of the cell cytoskeleton [reviewed in Zimmerman, 1970]. In general, moderate pressure (200–600 atm) induces cell rounding, blocks cytokinesis, inhibits cell crawling and amoeboid movements, stops active translocation of organelles and cytoplasmic streaming, and disrupts chromosome movement. Elevated hydrostatic pressure also disrupts intercellular adhesions and induces the dissociation of tissue cells.

Landau [1959, 1960] showed that hydrostatic pressures of 600 atm or greater induced interphase mammalian cells to round-up over several minutes. For short-term (20-min) treatments at moderate pressure (300–600 atm), the effects were reversible. The cells respread upon pressure release and progressed normally through the cell cycle. Based on the extensive studies of Marsland and coworkers [1948, 1956, review 1970], Landau proposed that cell rounding under pressure was due to the reversible solation of cytoplasmic gels, which were believed to be responsible for the generation and maintenance of cell shape [Landau, 1960]. Arrays of microtubules and actin microfilaments are obvious candidates for the labile cytoplasmic gels envisioned by Marsland and Landau.

In this paper, we present the first study of the changes in assembly and organization of microtubules

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and actin microfilaments that occur during pressure-induced rounding of interphase mammalian cells. Microtubules [Salmon and Wadsworth, 1986] and microfilaments [Pollard and Craig, 1982] are known to be dynamic polymers in some form of equilibrium with cytoplasmic pools of subunits. In interphase cells, microtubules are assembled into a stellate array termed the cytoplasmic microtubule complex (CMTC). Actin microfilaments form a low-density mesh throughout the cytoplasm [Begg et al., 1983]. They are also anchored to the plasma membrane and occur in high concentration in the cortex of motile cells. In epithelial cells spread flat upon the substrate, bundles of actin filaments, termed stress fibers, extend between sites of cellular adhesion to the substrate [Burridge, 1986]. Actin filaments also underlie protrusions of the cell cortex such as microvilli and lamellipodia [Matsudaira and Burgess, 1982]. Interphase cells grown in culture at 37°C at atmospheric pressure normally spread out flat and form adhesions to the substrate and to adjacent cells. Cell spreading and motility depend both on microtubules and the actin filament components of the cell cytoskeleton.

In our studies, interphase BSC-1 epithelial cells, grown on coverslips, were fixed under pressure using a pressure fixation chamber modified from the initial design of Landau and Thibodeau [1962]. Changes in cell morphology were observed by transmission and scanning electron microscopy, and the corresponding changes in the organization of microtubules and actin microfilaments were analyzed using fluorescent molecular probes and fluorescence microscopy.

**MATERIALS AND METHODS**

BSC-1 epithelial cells were grown to confluency at 37°C on 18 × 9-mm glass coverslips in Dulbecco's modified Eagle's medium supplemented with 20 mM HEPES, 10% fetal bovine serum, and antibiotics, pH 7.3. In preparation for the experiments, all solutions and specimen chambers were warmed to 37°C, and the large pressure bomb, or outer chamber, was kept in a 37°C water bath for the duration of the experiment (Fig. 1).

Cells were fixed under pressure using a modification of the pressure fixation apparatus designed by Landau and Thibodeau [1962], which is diagrammed in Figure 1. The apparatus was assembled as follows: two 18 × 9-mm coverslips were placed back-to-back in a stainless steel coverslip holder that was capped with an annular screw cap. The coverslip holder was then placed in the lower (or specimen) compartment of the inner chamber, which had been previously filled with 37°C culture medium. The upper compartment of the inner chamber contained double-strength fixative and a small stainless steel ball. The upper and lower compartments were separated by a #1 18-mm diameter circular coverslip. The upper compartment was screwed into the lower compartment to compress an annular rubber gasket against the circular coverslip and seal the upper compart-
ment to the lower compartment. It was important to prevent entrapment of air within either of these compartments during the set-up procedure. The entire inner chamber was placed into an outer chamber, or pressure bomb, as shown in Figure 1. The pressure bomb was filled with 37°C tap water and connected through a high pressure valve to a hydraulic pump. The entire chamber system was maintained at 37°C using a water bath. The rubber diaphragms on the bottom and top of the inner chamber transmitted the pressure in the bomb into the lower and upper compartments of the inner chamber.

Pressure was increased over several seconds, using the hydraulic pump. The bomb was then sealed with the high-pressure valve and disconnected from the pump. After 30 min the cells were fixed under pressure by vigorously shaking the entire bomb, which caused the steel ball to smash the circular coverslip. The ball and specimen holder then slid back and forth within the inner chamber; this resulted in mixture of the fixative with the cells. After fixation for 15 min, the coverslips were removed and processed as described below.

For electron microscopy, cells were fixed at a final fixative concentration of 2% glutaraldehyde in PME buffer (100 mM PIPES, 2 mM EGTA, 1 mM MgSO₄, pH 6.9). Coverslips were then removed from the pressure bomb, rinsed in PME buffer, and postfixed with 2% OsO₄ in PME. For scanning electron microscopy (SEM) observations, specimens were dehydrated in ethanol, dried in a Sorvall critical point drying apparatus, rotary coated with platinum/palladium metal in a Denton vacuum evaporator, and viewed using an International Scientific Instruments DS-130 scanning electron microscope.

Following osmication, coverslips for transmission electron microscopy (TEM) observations were dehydrated in ethanol and flat embedded in epon/araldite plastic. Thin sections were cut using glass knives on a Sorvall MT-2 ultramicrotome. Sections were stained with 5% uranyl acetate for 20 min, followed by 0.5% lead citrate for 7 min. Specimens were viewed with a Zeiss EM-10CA transmission electron microscope operated at 60 kV.

Microtubule arrays were visualized at the light microscope level using immunofluorescent staining. Cells were simultaneously lysed and fixed. Cells were lysed to reduce background staining from free tubulin subunits. The final concentration of the lysis/fixation solution was 80 mM PIPES, 5 mM EGTA, 1 mM MgSO₄, 0.5% Triton X-100, 2% paraformaldehyde, and 0.1% glutaraldehyde (pH 6.8). Following fixation in the pressure bomb, the coverslips were removed from the bomb and rinsed in phosphate-buffered saline (PBS: 0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH 7.3). Coverslips were then processed for immunofluorescence as described previously [Cassimeris et al., 1986].

Filamentous actin was localized with fluorescently labeled phalloidin. Cells were fixed under pressure at a final fixative concentration of 3.7% formaldehyde in PBS. Coverslips were removed from the pressure bomb and the cells were extracted in -20°C acetone for 2 min. The cells were rehydrated for 15 min. in PBS and then incubated for 30 min at 37°C with 25 µl of a 1:100 dilution of rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR).

Coverslips stained for microtubules or actin were rinsed with PBS and mounted in Gelvatol (Monsanto Co., St. Louis, MO). Cells were observed with a Zeiss 63 x/1.4 N.A. planapo lens on a Zeiss Universal microscope (Carl Zeiss, Thornwood, NY), equipped for epifluorescence, and photographed with Tri-X Pan film (Kodak), developed in Kodak HC-110.

RESULTS

We examined the effects of increasing pressure on interphase BSC-1 cells that had spread and grown to confluence on glass coverslips at 37°C and 1 atm pressure. Figures 2a and 3a show the typical morphology of these cells at atmospheric pressure. Cells were pressurized for 30 min and then fixed under pressure at pressures of 1, 180, 220, 290, 320, 400, 470, and 610 atm. Pressures of 290 atm or greater induced noticeable cell rounding and retraction from adjacent cells. The degree of retraction and rounding of the cells increased with increasing pressure, but at intermediate pressures (290, 320, and 430 atm), the degree of rounding was heterogeneous among the population of cells (see Fig. 2b). At 430 atm and above, almost all cells had retracted and were spherical or spindle-shaped. The great majority of cells at 610 atm detached from the coverslip during the fixation procedures.

At pressures of 290 atm and above, cell rounding was accompanied by a pronounced increase in the number of microvilli covering the cell surface (Fig. 3b). Thin sections through the cortex of pressurized cells showed fine, filamentous material aligned along the length of the core of the microvilli (Fig. 4). However, cross-linked bundles of actin filaments, as seen in the vertebrate intestinal brush border [Mooseker, 1985], were not visible.

The changes in the distribution and organization of actin filaments caused by high pressure were followed by labeling fixed cells with rhodamine-phalloidin. The organization of actin filaments in cells fixed after 30-min pressurization at 220 atm (Fig. 5a) appeared no different from controls fixed at atmospheric pressure (data not shown). Under both conditions, prominent stress fibers
Fig. 2. Scanning electron micrographs of interphase BSC-1 cells. 

a: Control cells fixed after 30 min at 1 atm in the pressure chamber. 
b: Cells pressurized to 430 atm for 30 min and fixed under pressure. 
c: Cells pressurized to 430 atm for 30 min and then released to 1 atm for 5 min before fixation. 
d: Cells pressurized to 430 atm for 30 min and then released to 1 atm for 30 min before fixation. Scale bar is 50 μm.
extended across the spread cells. Actin filaments were also concentrated at the cortex of the spread cells. At 290 atm (Fig. 5b), however, the number of prominent stress fibers was greatly reduced; they were fewer in number and thinner. The loss of stress fibers correlated with the morphological changes of cell rounding and
retraction. At 400 atm (Fig. 5c) and 470 atm (Fig. 5d), the cells were increasingly more contracted.

The disappearance of stress fibers at higher pressures did not result in a decrease in the intensity of phalloidin staining of the cells. Visually, the staining intensity increased with cell rounding and retraction (compare Fig. 5b with d). By focusing on the surface of the cell, punctate fluorescence was seen to be localized in microvilli distributed across the upper cell surface (data not shown). This observation indicates that pressure caused the actin in the stress fibers to be redistributed into microvilli, concurrent with cell rounding and retraction.

Elevated pressures exerted a distinctly different effect on the assembly of the microtubules in the CMTC. Although cells at 220 atm (Fig. 6a) appeared no different from controls, at 290 atm (Fig. 6b), the cells were noticeably retracted, but there was little apparent change in the density and average length of the microtubules. Above 290 atm, the density of microtubules decreased significantly. At 320 and 400 atm (Fig. 6c and d), anti-tubulin immunofluorescence revealed very few microtubules, and diffuse tubulin in the cytoplasm surrounded the nucleus. At 400 atm (Fig. 6d), only 10 to 20 long microtubules persisted per cell.

We tested the reversibility of these pressure effects by pressurizing cells at 400–430 atm for 30 min, releasing the pressure to atmospheric pressure, and then fixing preparations at various times after the pressure release. One hour after pressure release, the cells had respread substantially, the CMTC looked normal, and stress fibers reappeared in the cells (Fig. 7). However, the immediate
response to the pressure release was a pronounced contraction or further rounding of the cells (compare the cells in Fig. 2c with b), which preceded the slower respreading of the cells. This rapid contraction of mammalian cells upon pressure release was previously described by Landau [1960].

DISCUSSION

Our studies show that the normal distribution of both microtubules and actin microfilaments are reversibly disrupted by elevated hydrostatic pressures in the range that induces mammalian epithelial cells to retract and round-up. Microtubules persist at pressures that cause initial cell rounding, suggesting that disruption of microtubules is not the primary cause of rounding under pressure. Previous studies have shown that drug-induced microtubule depolymerization does not result in cell retraction and rounding [Vasiliev and Gelfand, 1976].

Our studies indicate that retraction and rounding coincide with the disruption of stress fibers. However, Sanger and coworkers have shown that disassembly of stress fibers either by inhibition of ATP production [Sanger et al., 1983], or by the natural disassembly that occurs during mitosis [Sanger and Sanger, 1980], is not sufficient to cause rounding. We have observed that, under pressures at which cells responded heterogeneously, isolated cells were more likely to round up than those in contact with adjacent cells (data not shown). Therefore, although cell retraction and rounding under pressure are probably functions of alterations in the assembly and organization of actin filament complexes,
Fig. 7. Pressure effects are reversible. Cells were pressurized to 430 atm in (a) and 400 atm in (b) and (c) for 30 min and then released to 1 atm for 1 hour before fixation. a: Scanning electron microscopy. b: Phalloidin fluorescence. c: Anti-tubulin immunofluorescence. Scale bar is 10 μm.

they must also be accompanied by the disruption of cell adhesions to the substrate and to other cells.

It is not surprising that treatment with 320 atm induced the depolymerization of the majority of microtubules in the CMTC. Similar pressures have been shown to induce the rapid disappearance of the majority
of polar spindle microtubules in a variety of cell types [Salmon, 1975a,b,c; Salmon et al., 1976] and of the labile cytoplasmic microtubules in the protozoa [Tilney et al., 1966]. Microtubule assembly in vitro has been shown to involve a large increase in molar volume, on the order of 100 ml/mol of polymerizing subunit [Salmon, 1975b]. Thus the depolymerization of the CMTc could be the result of direct effects of pressure on the tubulin association and dissociation reactions involved in the dynamic instability [Mitchison and Kirschner, 1984] and assembly behavior of the microtubules.

Microtubule assembly in vitro is much less sensitive to pressure than assembly in vivo. In vitro, at 37°C, 680 atm produces depolymerization of less than 15% of the tubulin assembled in the presence of a variety of microtubule-associated proteins (MAPs) [O'Connor et al., 1974; Salmon, 1975b; Engelborghs et al., 1976]. In contrast, 320 atm produces nearly complete depolymerization of the CMTc in vivo. Unfortunately, studies on the effect of pressure on the assembly of pure tubulin are not available; buffer conditions or stabilization by MAPs may account for the difference in stability observed for microtubules in vitro and in vivo.

Of particular interest is the abrupt depolymerization of the majority of microtubules between 290 atm and 320 atm of pressure. At 290 atm the number of microtubules per cell does not appear different from control cells. By increasing the pressure only 30 atm, there is a dramatic reduction in the number of microtubules per cell. This apparent critical pressure for microtubule depolymerization, coupled with the difference in the stability of microtubules in vitro and in vivo in response to pressure, suggests that pressure acts to depolymerize microtubules in vivo primarily by altering cellular regulatory mechanisms that control the nucleation and extent of microtubule assembly. Pressure may only secondarily affect microtubule polymerization reactions.

As with the CMTc, it is likely that the pressure-induced changes in the organization of actin filament arrays are indirectly produced by alterations in one or more regulatory mechanisms. The changes in the assembly state of actin filaments under pressure was surprisingly complex. The stress fiber disappearance occurred concurrently with the formation of cortical microvilli. Clearly, pressure did not induce overall depolymerization of actin filaments in the cell. The polymerization of purified actin in vitro is only slightly reduced by 600 atm pressure [Swezey and Somero, 1982; Begg et al., 1983]. In contrast, pressure as low as 290 atm induces the disappearance of stress fibers. This must be the consequence of the depolymerization of actin filaments in the stress fibers, due to pressure effects on actin-associated complexes or regulatory processes more sensitive to pressure than the polymerization of purified actin in vitro. Conversely, actin filaments in the stress fibers and in the cortex of cells at atmospheric pressure may redistribute without disassembly into microvilli as the cell rounds-up under pressure.

Pressure may alter the cellular regulatory mechanism(s) that control the changes in cytoskeletal assembly during the cell cycle. This hypothesis is based on the striking similarity between the changes in cell shape and cytoskeletal organization produced by pressurization and those that occur when interphase tissue cells enter mitosis [Harris, 1973; Erickson and Trinkaus, 1976; Sanger et al., 1984]. Interphase cells typically adhere to each other and to the underlying substrate by adhesion sites at the periphery of the cell. At the interphase-mitosis transition, these cells retract and round-up. As a cell rounds-up, these adhesion sites remain attached to the cell body by thin cytoplasmic strands or retraction fibers. Concurrent with cell rounding, the CMTc and stress fibers disappear and numerous microvilli, 0.5 to 1 μm long, appear on the cell surface. These morphological and cytoskeletal changes are identical to those we observed when interphase cells are pressurized to 320 atm or greater. The regulatory mechanisms that produce the morphological and cytoskeletal changes at the interphase-mitosis transition are still not understood. Phosphorylation of specific proteins or elevation of cytosolic calcium have both been proposed as regulators of cell cycle transitions [Vandre et al., 1984].

Calcium may also be involved in the regulatory pathway that is affected by pressure when cells round up. Elevated pressure may induce a rise in cytosolic Ca²⁺ concentration, which in turn inhibits microtubule polymerization, disrupts actin filament assembly, and severs the cross-bridges that hold cortical actin filaments in a gel [Otter et al., 1987]. There are as yet no direct measurements to support the hypothesis that pressure induces a rise in cytosolic Ca²⁺, but several indirect observations make this hypothesis feasible. First, microtubule assembly in vivo is highly sensitive to micromolar levels of calcium [Kiehart, 1981; Keith et al., 1983; Izant, 1983]. Secondly, actin gels in vitro are solated either by disruption of actin filament cross-links or by the severing of filaments into short lengths. The actin-binding proteins associated with either process are known to be calcium-sensitive: micromolar levels of calcium can inhibit cross-bridging and can induce filament severing [Stossel et al., 1985; Sanger et al., 1987]. Calcium-sensitive cross-bridging proteins such as α-actinin, and severing proteins such as gelsolin, are known to be associated with stress fibers [Sanger et al., 1987].

Calcium has also been implicated as the mediator
in the response of paramecia to high pressure [Otter and Salmon, 1979, 1985; Otter et al., 1987]. Observations of the movements of swimming paramecia, Ni2+-paralyzed cilia, and ATP-reactivated, detergent-extracted cells under pressure led to the conclusion that the application of moderate hydrostatic pressure produces a small rise in intracellular Ca2+. In addition, pressure is known to inhibit the ATPase activity of the calcium pump in the sarcoplasmic reticulum [Champeil et al., 1981] and to induce contraction of muscle [Hogan and Dahl, 1987].

Our results suggest that pressure-induced disruption of the cytoskeleton is due to effects on regulatory mechanisms rather than direct effects on the polymerization reactions of microtubules or actin. These may be the same mechanisms that regulate the interphase-mitosis transition during the cell cycle. Further high-pressure studies of the factors responsible for cytoskeletal disruption and cell rounding may lead to important information on the regulation of the interphase-mitosis transition.

In addition, high-pressure studies will yield important information on the cytoskeletal adaptations of deep-sea organisms. Organisms have been found living at ocean depths exceeding 10,000 meters, which would correspond to over 1,000 atm of pressure. Clearly, these organisms have evolved molecular mechanisms, different from mammalian cultured cells, which enable them to retain their structure and cellular function at high pressure. These differences may be in the polymer subunits themselves, in associated proteins, or in regulatory complexes. The experiments reported here are the foundations for further examination of the differences between the molecular mechanisms regulating the cytoskeletal dynamics of deep-sea and terrestrial organisms.

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