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Transient Loss of αB-Crystallin: An Early Cellular Response to Mechanical Stretch

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Human trabecular meshwork (HTM) is distended and stretched with increases in intraocular pressure. During this stretching, there is a rearrangement of actin filaments. The HTM cells express αB-crystallin, a small heat shock protein that may have a role in the stabilization and regulation of the cytoskeleton in mammalian cells. The levels of αB-crystallin were examined in trabecular meshwork cells after mechanical stretch. Human TM primary cell cultures, plated onto silicone sheets, were subjected to a single 10% linear stretch and samples were prepared at various times after stretch for immunoblotting or Northern blotting. Immunoblots of total protein extracts with antibody specific for αB-crystallin detected a 26% decrease of cellular αB-crystallin levels within 2 minutes. After 1 hour αB-crystallin levels had decreased 90% compared to control cells. The levels of αB-crystallin began to recover in cells stretched for 2 hours and returned to initial levels by 24 hours. Northern blots probed with αB-crystallin exon III cDNA detected a transcript of 0.65 kb in human TM cells and the levels of the αB mRNA remained constant during αB-crystallin protein decrease. Later, levels of the 0.65 kb transcript of αB-crystallin increased during the cellular recovery. These results suggest that decreased levels of αB-crystallin after mechanical stretch were probably not due to transcriptional changes but rather to increased degradation of αB-crystallin protein. An increase in mRNA levels may play a role in the recovery of αB-crystallin during reorganization of the cytoskeleton and attachment to the substratum. These data raise the possibility of a specific proteolysis of αB-crystallin protein in cells after a physiological challenge. © 1997 Academic Press

Human and monkey trabecular meshwork cells express αB-crystallin, a small heat-shock protein (sHSP) related to HSP27, which is inducible by heat shock and oxidative stress (1, 2). The α-crystallins are thermostable proteins that display molecular chaperone activity as characterized by their ability to prevent thermally-induced aggregation of other crystallins and proteins *in vitro* (3). While αA-crystallin expression is confined largely to lens, αB-crystallin is expressed in many tissues including heart, brain, spinal cord, lungs, skeletal muscle, and skin where its cellular function is not known (4, 5). Increased expression of αB-crystallin is also induced by Cd^{2+}, and sodium arsenite in NIH 3T3 cells, Co^{2+} and heat-shock in astrogliomal cells, and by hypertonic stress in dog lens epithelial cells (6-8). Like HSP27, αB-crystallin monomers (~24 kDa) form supramolecular structures with sedimentation values of about 17S that are normally cytoplasmic but associate with the nucleus at high temperatures (6).

There is evidence that sHSPs associate with the cytoskeleton (CSK) (9-11). Affinity chromatography has been used to demonstrate the association of α-crystallins with actin and desmin filaments *in vitro* (12, 13). The lens cell intermediate filament proteins CP49 and CP115 form beaded filaments with α-crystallin *in vitro* similar to those from lens fiber cells (14). Double-labeling immunofluorescence of lens epithelial cells, and ultrastructural examination of fiber cells suggested some α-crystallin association with actin (15, 16). Recently, it was demonstrated that the α-crystallins (both αA-and αB-crystallin) stabilize actin filaments and slow the depolymerization of actin (17). In that report non-phosphorylated α-crystallins were shown to be superior to phosphorylated forms as inhibitors of the cytochalasin-D induced depolymerization of skeletal muscle actin filaments *in vitro.*

Aqueous humor flows out of the eye through the trabecular meshwork (TM) and into Schlemm's canal. As intraocular pressure increases, the TM is distended and stretched. When the TM is distended, actin reorganization occurs, and some drugs that increase the outflow of aqueous humor also alter actin filaments (18,

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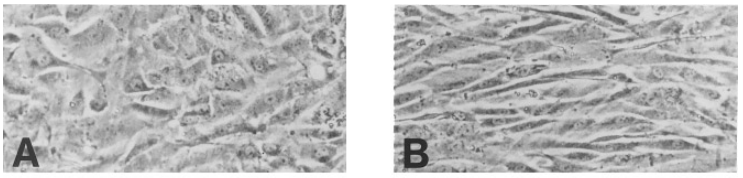

A. Control Cells
B. Cells Stretched by 10%

FIG. 1. (A) Human trabecular meshwork (TM) cells plated onto silicone sheets coated with laminin, before mechanical stretch. Well defined nuclei are visible under phase contrast optics. (B) Human TM cells immediately after 10% stretch. Cells remained attached to the silicone and recovered their pre-stretch morphology within 24 hours.

19). If αB-crystallin plays some role in the modulation of the CSK then perturbations of the CSK may alter its levels or phosphorylation state. Since the TM is a region critical to the aqueous outflow, we have attempted to model a system *in vitro* to study the stretching process. We have therefore examined αB-crystallin protein levels, mRNA levels, and phosphorylation state after mechanical stretch of primary cultures of human TM cells.

MATERIALS AND METHODS

Primary human trabecular meshwork (HTM) cultures were established from human donor eyes obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) within 48 hours of death. The dissection and explant preparation was similar to those previously described (19, 20). Primary cultures from three different donors were used. Cells were maintained at 37°C in Dulbecco's minimal essential medium (DMEM) containing 20% fetal calf serum (Gibco-BRL; Gaithersburg, MD). Cells were plated onto boats formed from silicone sheets, previously coated with laminin, attached to end supports that were anchored into a support frame (21). Notches in the support frame were available to reset boats to a 10% linear stretch. Cells were plated at 1×10^6 cells/sheet, 48 hours before experiments, and the media was changed to serum-free DMEM 24 hours before mechanical stretching. The silicone sheets were stretched and maintained in the stretched position for various periods of time.

After stretch, cells were quickly washed with PBS and scraped off the silicone boats at selected times in 10 mM Tris-HCl (pH 7.0) 1.0% Triton X-100, which was found to completely solubilize cellular αB-crystallin. Protein concentrations were determined with the Bradford assay (22). Some cells cultures were also extracted in 10 mM Tris-HCl pH 7.4, 50 mM NaF, 0.14 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA to obtain buffer soluble and buffer insoluble fractions. Protein (2μg) was subject to SDS-PAGE or two-dimensional gel electrophoresis on 12.5% Phastgels using the Pharmacia PhastGel System (Pharmacia LKB, Piscataway, NJ). Polypeptides were transferred to nitrocellulose membranes as per the manufacturer's protocols and blocked for 2 hours. Membranes were incubated with rabbit polyclonal antibody to αB-crystallin overnight at 4°C. The αB-crystallin antibody was a gift from Dr. J. Horwitz (Jules Stein Eye Institute) and is specific for a 13-mer polypeptide corresponding to the c-terminus of bovine αB-crystallin that does not share sequence homology with any heat shock proteins (23). Blots were incubated with goat anti-rabbit IgG alkaline phosphatase conjugate and then developed with CSPD chemiluminescence substrate (Tropix, Inc., Bedford MA). Densitometry was carried out using the Umax Powerlook II scanner (Umax Data System, Inc., Taiwan, R.O.K.) and the data was analyzed using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Two separate experiments were done for each time point.

Northern blots were prepared by standard capillary transfer of total cellular RNA from formaldehyde agarose gels onto nylon membranes (Boehringer Mannheim, Indianapolis IN). Membranes were washed in 6×SSC for 5 minutes and RNA was cross linked using a UV Stratalinker 2400 (Stratagene, La Jolla CA). For αB-crystallin mRNA, a cDNA corresponding to most of exon III of αB-crystallin (2849-3092) was used and blots were subsequently stripped and reprobed with cDNA (1 kb) specific for the 18S RNA. Non-radioactive digoxigenin (DIG)-labeled probes were prepared by random priming using the DIG High Prime DNA Labeling and Detection Kit (Boehringer Mannheim). Membranes were blocked at 42°C in hybridization buffer composed of 7% SDS, 50% formamide, 5×SSC, 2% blocking reagent (Boehringer Mannheim), 50 mM sodium phosphate, ph 7.0, 0.1% N-lauroylsarcosine, and hybridized with the appropriate DIG-labeled DNA probe in the same buffer (42°C). Blots were washed in 2×SSC, 0.1 % SDS (2×15 min, 25°C) followed by 0.5×SSC, 0.1% SDS (2×20 min, 54°C) to remove unbound probe. Densitometry was done as described above. Three separate determinations were done for each time point.

RESULTS

HTM cells subjected to a 10% mechanical stretch remained attached to their substratum and displayed little increase in cell death compared to non-stretched controls. The viability of cells stretched at 10% for 2 hours was 88% ± 4% and for 24 hours was 81% ± 6%. The stretch of the cells resulted in a visible elongation of the cell bodies that gradually returned to pre-stretch morphology over a period of 24 hours (Figure 1).

Immunoblotting of protein extracts with antibody specific for αB-crystallin detected a single band at the expected molecular mass of around 24 kDa (Figure 2A). The αB-crystallin levels of HTM cells were decreased 26% percent within 2 minutes after a 10% mechanical stretch and within 1 hour the decrease was 90% (Figure 2B). By 6 hours after stretch the levels of αB-crystallin were starting to return to pre-stretch levels. After 24 hours, when HTM cells had regained a morphology indistinguishable from unstretched cells, αB-crystallin had increased to control levels. Examination of the 1% Triton X-100 insoluble fraction by immunoblotting

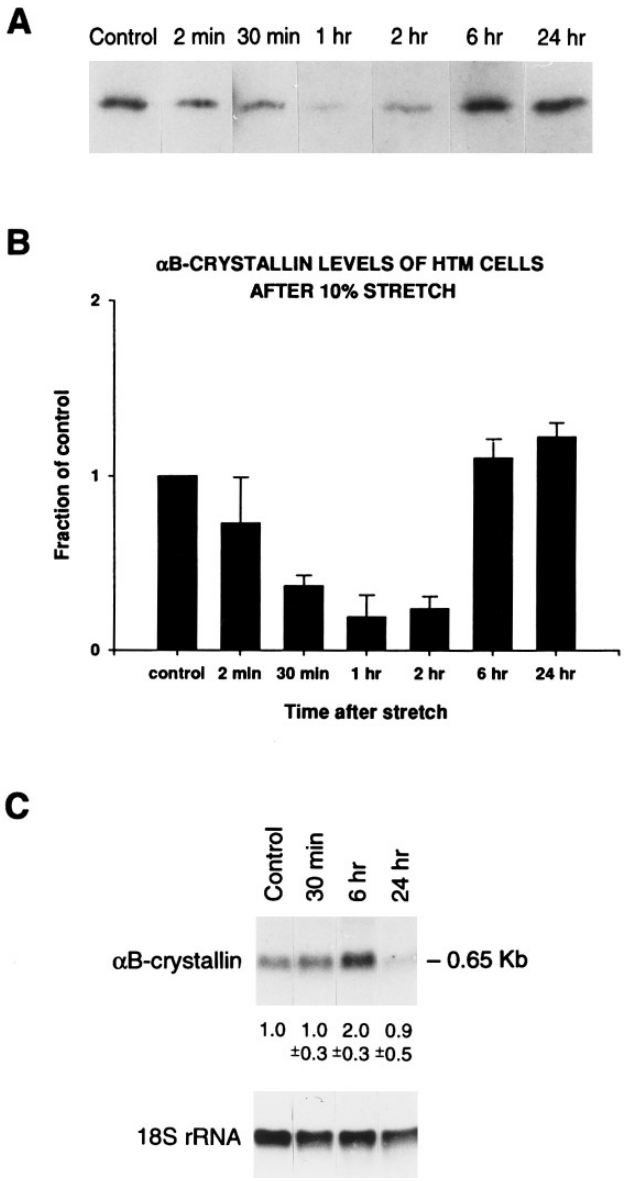

FIG. 2. (A) One-dimensional immunoblots of human TM cell total protein (2 μg) probed with antibody to αB-crystallin detected a 24 kDa band after mechanical stretch. Control cells were not stretched. (B) Graphical representation of the results of densitometric scan and analysis of the chemiluminescent bands of αB-crystallin in human TM cells after mechanical stretch. The values are normalized to the control value. (C) Northern blots of human TM cell total RNA hybridized with probe to αB-crystallin detected a 0.65 kb transcript. Blots were stripped and reprobed for 18S rRNA to equalize density measurements. Samples represent the phases of αB-crystallin loss (30 min), increase (6 hr), and completion of cell adaption to stretch (24 hr). Control cells were not stretched. Total band densities normalized to the control level are shown.

failed to detect αB-crystallin (data not shown). The αB-crystallin was detected only in the buffer soluble phase either with or without detergent. Northern blots of total cellular RNA were hybridized with αB-crystallin probe and a single major transcript was detected of 0.65kb in length (Figure 2C). Transcript levels did not decrease during the time of αB-crystallin loss and remained constant through the 1 hour post-stretch period. During the recovery phase of αB-crystallin, 6 hours after stretch, the αB-crystallin 0.65 kb transcript level was double that of the control. Levels of the 0.65 kb transcript then returned to control levels by 24 hours after stretch.

Immunoblots of two-dimensional gels were examined with antibody to αB-crystallin (Figure 3). The two-dimensional gel pattern for trabecular meshwork cells and the immunoblots corroborated the data obtained by the one-dimensional gels and at all time periods a single strong major spot was detected corresponding to the known position of non-phosphorylated αB-crystallin. The silver stained two-dimensional gels from the control, 2, 15, and 60 minute time points had nearly identical patterns. Immunoblotting of an isoelectric focussing gel comparing HTM cell αB-crystallin with human lens soluble crystallins confirmed that HTM cell αB-crystallin had the same isoelectric point as non-phosphorylated αB-crystallin. (Figure 3B).

DISCUSSION

Primary cultures of HTM cells were elongated by mechanical stretch which displaced their CSK. HTM cells responded by gradually adapting their morphology back to the initial state within 24 hours. A 10% linear stretch caused no visible detachments or damage compared to non-stretched cells as assessed by the trypan blue exclusion assay. This is not surprising since TM cells are known for their ability to migrate and attach in culture and on the trabecular beams of the eye *in vivo* (24, 25).

Immunoblots for αB-crystallin revealed a dramatic response to mechanical stretch in the form of a transient decrease of this protein. This rapid decrease in αB-crystallin levels represents a novel regulatory response that contrasts the increase in αB-crystallin after heat- shock, heavy metals, arsenite and hypertonicity and the slow loss over days of the αB-crystallin after denervation of the adult rat hindlimb muscle (2, 6-8, 26). A 90% loss of initial αB-crystallin content occurred within 1 hr after stretch and the ensuing recovery of αB-crystallin occurred on a time scale consistent with the recovery of cell morphology to its initial state. In contrast to protein denaturing stresses such as heat-shock, examinations of soluble and insoluble fractions from stretched HTM cells did not detect transfer of the αB-crystallin from the buffer soluble to the insoluble phase. This suggests that αB-crystallin's CSK-related functions are distinctly different from the heat shock response that collapses αB-crystallin and intermediate filaments around the cell nucleus (6, 27).

Several possibilities for the loss of αB-crystallin must be considered. Covalent cross linking of αB-crystallin

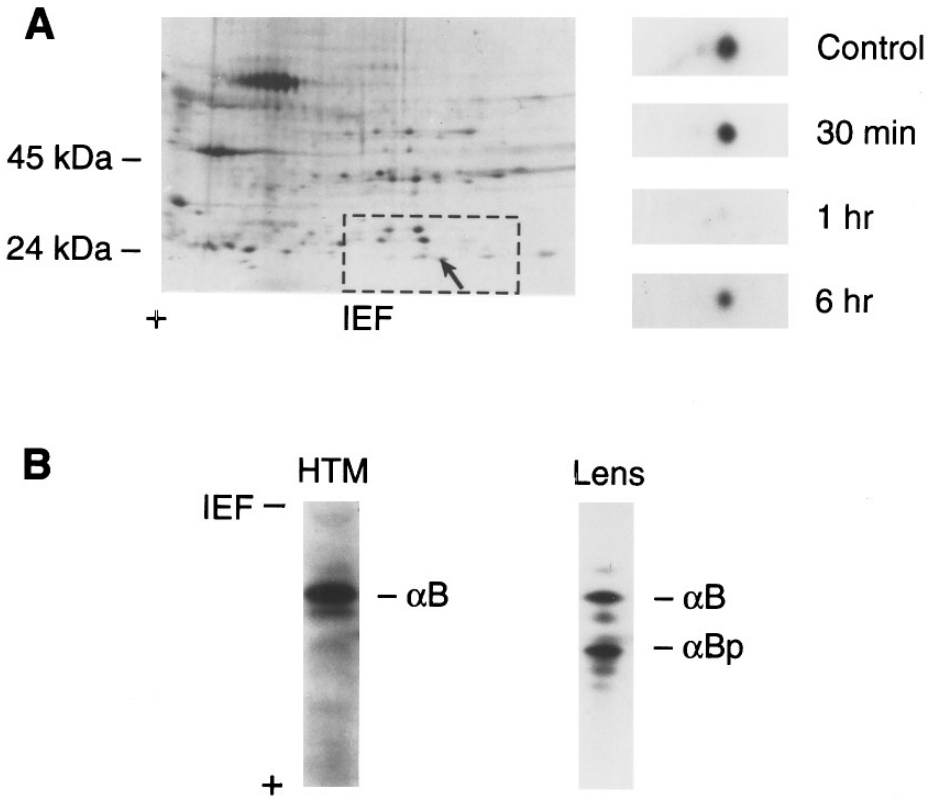

FIG. 3. (A) Immunoblots (right) from two-dimensional gels of human TM cells after mechanical stretch probed with antibody to αB-crystallin. A single major spot was detected corresponding to the known position of the non-phosphorylated form of αB-crystallin (arrow) on silver stained gels of human TM cell soluble protein. (B) Immunoblots of IEF gels comparing human TM cell soluble protein and human lens crystallins probed with antibody to αB-crystallin: αB, non-phosphorylated; αBp, phosphorylated. The immunoblot was overexposed to show the phosphorylated form of αB-crystallin.

into an SDS/β-mercaptoethanol insoluble complex is not very likely. There was no indication on the immunoblots of higher molecular weight forms of αB-crystallin that would be anticipated if covalent cross linking was rapidly occurring. Decreases in αB-crystallin levels could result from diminished synthesis; however, northern blot analysis showed that mechanical stretch did not cause a decrease of αB-crystallin transcript during the loss of αB-crystallin protein. If decreased translation caused the loss of αB-crystallin then the 26% decrease of this protein within 2 minutes of stretch would suggest an extremely short half life. Accumulation of αB-crystallin in the lens or in Alexander's disease would not appear to support the idea of a short half-life for this protein (28). Another possibility would be the leakage of αB-crystallin and other proteins from the cell. The nearly identical two-dimensional gel patterns of the control and 1 hour time points would suggest that selective leakage of proteins with certain molecular weights or isoelectric points does not occur. However, a specific loss of αB-crystallin to the medium cannot be ruled out at this time. Immunoblotting and northern data appear most consistent with a proteolytic loss of αB-crystallin. Ubiquitin-dependent protein degradation could be a possible mechanism for a regulatory removal of αB-crystallin after stretch. Alpha-crystallins are non-"N-end rule" substrates for the rabbit reticulocyte and bovine lens epithelial cell ubiquitination systems *in vitro* (29-31). Furthermore, αB-crystallin appears to be ubiquitinated in glial cells (32). There is evidence that ubiquitin conjugating enzymes are involved in the regulatory degradation of non-"N-end rule" proteins such as p53 and are involved in G_1-S cell cycle progression (33, 34). Proteins that modulate the CSK are also candidates for degradation by the ubiquitin/proteosome system such as the microtubule-binding protein Ase1 that is required for spindle elongation and separation of the spindle poles during mitosis. Non-degradable ubiquitination-resistant mutants of Ase1 delay spindle disassembly and prevent cell entry into G_1 (35).

Phosphorylated forms of αA- and αB-crystallin are present in the lenses of different species; however, the mechanism of phosphorylation and the possible function of this post-translational modification are unknown. Alpha-crystallins can be phosphorylated by cAMP dependent kinase *in vitro,* and some autophosphorylation can be detected by radiolabel *in vitro* (36,

37). Phosphorylation of αA- and αB-crystallin has no effect on the inhibition of GFAP or vimentin intermediate filament assembly *in vitro* (38). While HSP27 is phosphorylated in Chinese hamster cells after heat shock, αB-crystallin is not phosphorylated after heat shock in human gliomal cells or NIH 3T3 cells (6, 7, 39). Examination of HTM cell extracts by two-dimensional immunoblots revealed one major non-phosphorylated αB-crystallin. We conclude that there is little detectable modulation of αB-crystallin phosphorylation state during stretch-induced CSK remodeling. It still possible that phosphorylation may occur if such intermediates are rapidly degraded.

The HTM cells *in vivo* undergo alterations in their CSK with increases in intraocular pressure as well as with some drugs that increase the outflow of aqueous humor. Understanding the steps involved in the rearrangement of the actin filaments and the CSK would be an important step in determining regulation of intraocular fluid and pressure. The CSKs of neighboring cells and the underlying ECM are interconnected by junctional complexes and focal adhesion complexes so that stretching of the ECM transmits force into the entire network. The balanced tension of the CSK generated by the action of actin/myosin type filament sliding is termed tensegrity (40). Application of an external force changes the tensegrity equilibrium, altering the CSK and its potential interactions with regulatory and signal transduction components. The temporal correlation of transient αB-crystallin loss and cell shape changes after stretch in the HTM cells suggests that αB-crystallin may play an important role in CSK stability and remodeling.

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