SWELLING-INDUCED ACTIN REORGANIZATION FACILITATES REGULATORY VOLUME DECREASE IN ANULUS FIBROSUS CELLS

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INTRODUCTION

Long term mechanical loading on the spine is one of the factors implicated in degeneration of the intervertebral disc (IVD). Under normal physiologic conditions, the IVD is subjected to static and dynamic compressive loads that lead to diurnal variations in disc height and water content [1]. As the disc is loaded and unloaded, water is expressed from the tissue and reabsorbed leading to exposure of embedded cells to both hyper and hypo-osmotic stresses. Because the cell membrane is relatively permeable to water, changes in extracellular osmolality lead to rapid changes in cell volume. In some cell types, alterations in cell volume trigger volume recovery mechanisms that involve the activation of ion channels and the initiation of intracellular signaling cascades [2].

The actin cytoskeleton plays a key role in maintaining the shape and structural integrity of the cell. Recent studies suggest a role for the actin cytoskeleton in the cellular response to hypo-osmotic stress [3]. Cell swelling leads to depolymerization of the F-actin network in a number of cell types [4]. This reorganization of the F-actin network may be necessary to facilitate the transport of solutes and water into and out of the cell after exposure to hypo-osmotic stress. As such, the actin cytoskeleton may play a significant role in the control of regulatory volume decrease (RVD) after cell swelling. This hypothesis is supported by evidence that the stabilized F-actin network may act as a barrier to small molecule diffusion [5].

The objective of this study was to investigate the hypothesis that isolated cells from the anulus fibrosus (AF) of the IVD respond to hypo-osmotic stress by swelling and initiating regulatory volume mechanisms. Further, the presence and role of F-actin breakdown and reorganization in cell swelling and RVD was examined.

MATERIALS AND METHODS

Cell Culture. AF tissue was harvested from porcine lumbar spines shortly after sacrifice. Cells were released by a sequential pronase/collagenase digestion and cultured in DMEM/F-12 medium containing 10% fetal bovine serum at 37°C and 5% CO_2.

Osmotic Stress. Solutions of varying osmolality (150-300 mOsm) were prepared by diluting a physiological salt solution (310 mOsm) with distilled water.

Actin Modulation. Cytochalasin D (2 μM, Sigma) was used to disrupt, and Phalloidin (1 μM, Molecular Probes) to stabilize F-actin. Cells were treated with these agents for 3 hours prior to testing.

Actin Quantification. Isolated AF cells in suspension were exposed to solutions of varying osmolality (150-300 mOsm) for 1 and 10 minutes and then fixed in 3.7% formaldehyde in a solution of the same osmolality. Cells were permeabilized and the actin stained in a single step using a solution containing 0.1% Triton X-100, 1% BSA, and 0.3μM rhodamine phalloidin (Molecular Probes) in PBS. Fluorescence intensity was assayed by flow cytometry and normalized to iso-osmotic control values.

Actin Organization. Cells were labelled with rhodamine phalloidin as described above and visualized using a confocal laser scanning microscope (LSM 510, Zeiss, Inc.). The organization of the F-actin network was described by comparing actin at the cell membrane with that found in the interior of the cell. To quantify changes in actin organization, a ratio of fluorescence intensity at the membrane to that in the cytosol was reported [6].

Cellular Volume Change. Cell volume measurements were performed using a custom-written digital image analysis program [7]. In order to quantify the rate of cell swelling, an exponential fit was performed to the volume data from the time of application of osmotic stress to the time at which peak volume was obtained to determine a swelling time constant ($\tau_{swelling}$). A decreasing exponential model was used to fit the RVD portion of the volume curve to determine the recovery time constant (τ_{RVD}).

RESULTS

Control experiments confirmed that exposure to an iso-osmotic solution did not result in volume change or actin breakdown in AF cells. Application of hypo-osmotic stress to isolated cells gave rise to a decrease in total cellular F-actin at 1 minute followed by a return towards baseline after 10 minutes (Figure 1). Significant differences were observed only at 150 mOsm. All 3 magnitudes of hypo-osmotic

stress gave rise to a significant decrease in the cortical component of F-actin at 1 minute (data not shown). A trend toward recovery of the cortical actin localization was observed by 10 minutes but was not significant. AF cells swelled proportionately to the applied osmotic stress with the greatest volume change occurring after exposure to 150 mOsm (Figure 2a). Pre-treatment of cells with Cytochalasin D to disrupt the F-actin did not affect the extent of cell swelling (Figure 2a). Significant RVD was observed at all levels of osmotic stress, but the extent of RVD was similar across all groups (Figure 2b). Pre-treatment with Cytochalasin D gave rise to a significant decrease in the extent of RVD compared to untreated cells at all levels of osmotic stress (Figure 2b). The rate of cell swelling ($\tau_{swelling}$) was similar for all levels of osmotic stress and was unaffected by Cytochalasin D treatment (Figure 3a). The time constant for RVD (τ_{RVD}) however, decreased significantly with increasing osmotic stress and was also unaffected by Cytochalasin D treatment (Figure 3b). It was not possible to describe the effect of phalloidin treatment on cellular volume adaptation as significant cell blebbing occurred at all levels of osmotic stress, making accurate determination of cell volume impossible.

DISCUSSION

The results of this study support the hypothesis that isolated AF cells from the IVD rapidly swell upon exposure to hypo-osmotic stress and then exhibit RVD toward baseline cell volume. Further, this study suggests that the F-actin cytoskeleton plays a key role in controlling cellular volume change after exposure to hypo-osmotic stress. The observed dose-dependent response to hypo-osmotic stress of both F-actin breakdown and the rate of cellular RVD suggests that disruption of the cortical actin network is a key step in volume recovery, but not in the initial cell swelling. It has been suggested that the mechanism of this relationship may be mechanical as a stabilized F-actin cortex has been shown to act as a barrier to the transport of small molecules [5]. However, the diminished RVD observed after Cytochalasin D treatment suggests that simple disruption of the actin barrier is not sufficient for effective RVD. Rather, active depolymerization followed by reorganization of the F-actin network appears to be necessary for functional RVD. This observation is supported by studies which suggest that the actin cytoskeleton modulates volume recovery through its physical interactions with key transport proteins in the cell membrane [3]. Previous studies have shown that the response of isolated AF cells to hypo-osmotic stress includes the generation of intracellular calcium transients [8]. In light of this finding, the actin changes observed in this study may play a role in a multi-step mechanism leading to effective volume recovery after hypo-osmotic stress. In conclusion, our findings implicate the F-actin cytoskeleton of AF cells as a potentially important transducer or effector of osmotic and mechanical stresses in the disc.

ACKNOWLEDGMENTS

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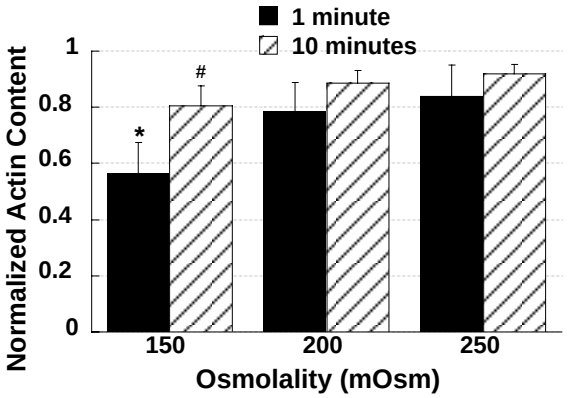

Figure 1: Hypo-osmotic stress leads to F-actin breakdown in AF cells after 1 minute followed by a recovery at 10 minutes. *p<0.05 vs. iso-osmotic control, # p<0.05 vs. 1 minute group of same osmolality

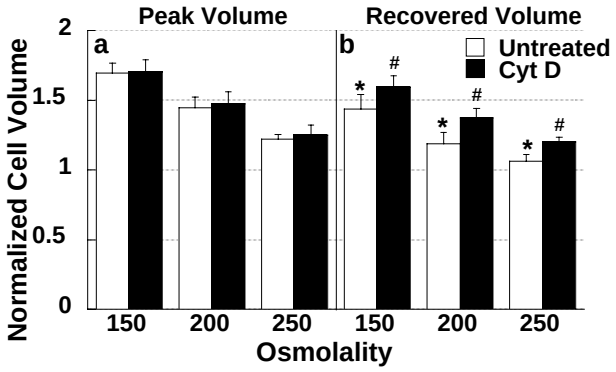

Figure 2: (a) AF cells swell after hypo-osmotic stress in a dose-dependent fashion. (b) Cell swelling was followed by RVD which was diminished after pre-treatment of cells with Cytochalasin D. *p<0.05 vs. peak volume, #p<0.05 vs. untreated recovered volume.

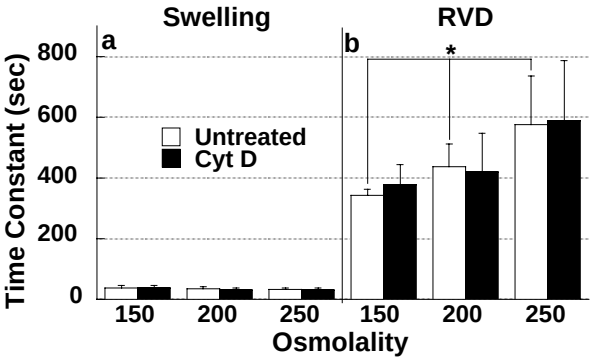

Figure 3: (a) All cells swelled rapidly at a similar rate ($\tau_{swelling}$) after hypo-osmotic stress. (b) The rate of cellular RVD (τ_{RVD}) was greater at higher magnitudes of hypo-osmotic stress, and was unaffected by Cytochalasin D treatment. *p>0.05