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Realignment of f-actin Cytoskeleton in Osteocytes after Mechanical Loading

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Abstract**—**F-actin fibrils are the cytoskeleton of osteocytes. They react in a dynamic manner to mechanical loading, and strength and reposition their efforts to reinforce the cells structure. We hypothesize that f-actin is temporarly disrupted after loading and re-polymerizes in a new orientation to oppose the applied load. *In vitro* studies are conducted to determine f-actin disruption after varying mechanical stimulus parameters that are known to affect bone formation. Results indicate that the f-actin cytoskeleton is disrupted *in vitro* as a function of applied mechanical stimulus parameters and that the f-actin bundles reassemble after loading induced disruption within 3 minutes after cessation of loading. The disruption of the f-actin cytoskeleton depends on the magnitude of stretch, the numbers of loading cycles, frequency, the insertion of rest between loading cycles and extracellular calcium. *In vivo* studies also demonstrate disruption of the f-actin cytoskeleton in cells embedded in the bone matrix immediately after mechanical loading. These studies suggest that adaptation of the f-actin fiber bundles of the cytoskeleton in response to applied loads occurs by disruption and subsequent re-polymerization.

Keywords**—**Mechanical loading of osteocytes, f-actin cytoskeleton, disruption, re-polymerization.

I. Introduction

THE cellular cytoskeleton is a scaffold found within the cytoplasm of all cells. It is a dynamic structure, which provides the cell with support, in order to maintain shape and organization [1]. Bone cells poses the ability to adapt to a changing workload and restructure their strength and density on a need-to basis. Bone cells respond more prominently to dynamic strains that change at high physiological rates than static ones. That is, osteocytes adapt more strongly to sudden bursts of high strains. These strains are varying in both intensity and frequency. Therefore, they are less typical than those strains produced by cyclic loads, such as cardiovascular exercises, which are generally more repetitive [2]. The application of fluid shear to the cell membrane of osteoblasts initiates the generation of the actin stress fiber, f-actin. With the application of 12 dyn/cm^2 for a duration of 60 min, actin filaments become visibly thicker and more abundant [3]. During in vitro studies, it has been determined that the f-actin cytoskeleton shifts from its original position and reorients itself to oppose the applied mechanical load [4]. The f-actin cytoskeleton fibrils first dissolve and then re-polymerize in the direction of their new orientation. In this response, the cell is acting to increase its elastic modulus in the direction of the applied load, and therefore reduce the strain that the cell endures [5]. This reaction allows the cell to resist changes in it's normal function; a common physiological response, which is referred to as homiostasis[6].

These dynamic strains cause an immediate disruption in the alignment of f-actin. Our data, presented in this paper, shows that following a disruption in the orientation of f-actin fibrils, a new orientation is organized within 3 minutes.

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II. Methods

MLO-A5 cells, a late-osteoblast, early-osteocyte cell-line, were used to conduct the in vitro studies. A lower stress magnitude was used to ensure that the cells would not de-adhere from the substrate. Additionally all of the experiments were performed in an incubator that was held at 37 °C and a carbon dioxide content of 5%. All cells were cultured in α-MEM (α- minimum essential medium) containing 5% bovine serum , 5% calf serum and 1% penicillin-streptomycin (Gibco, Gaithersberg, MD). Twenty-four hours prior to experimentation, the cells were plated at 40,000 cells per well, with approximately 7 cm^2 area and 3mL of media for each well. The cells reached 10-20% confluency before experiments.

Cells were grown on six-well silicone plates coated in collagen I that were loaded in equi-axial stretch on Flexcell 4000T (Flexcell International Inc., Hillsborough, NC). To apply loading to this system, the membrane is stretched over a central post by applying a vacuum just below the membrane and adjacent to the central post. In previous studies, it has been shown that uniform strains can be obtained from the portion of the membrane directly over the post. To ensure that the cells in our studies received uniform stress, all of the cells were taken from the region of the membrane over the post.

Using this membrane technique ensures that the stress applied to the membrane equals the stress applied to the cells.

Since this membrane technique induces an equal stretching in all directions of the membrane, it too induces an equal stretching in all directions of the cells. Hence, it is not necessary to keep tract of the orientation of the cells on the membrane. The cells are subjected to short durations of stretching, following which 5mL of cold, fresh, paraformaldehyde is poured into wells at intervals of 0, 15, 30, 45, 60, and 180 seconds following the application of stretch. This technique has been proposed to instantaneously fix proteins located within cells [7]. After fixation for 5 minutes, wells were washed twice in phosphate buffered saline (PBS), the cell membrane was then dissolved in 0.5% Triton X-100 (Sigma Aldrich, St. Louis, MO), and the wells washed again twice in PBS. The treated cells were stained using AlexaFluor Phalloidin (Molecular Probes/ Invitrogen, Carlsbad, CA), and coverslipped. . Cells with an intact and completely depolymerized cytoskeleton were counted under a microscope (Figure 3; Nikon TE-200, Nikon Corp.). Cells in located on the central region of the membrane, where strain is uniform, were categorized into cells with an intact f-actin cytoskeleton, a partially depolymerized cytoskeleton, and cells with a completely depolymerized cytoskeleton (shown in Fig. 1). Each experiment was repeated at least three times, with the results averaged and standard deviations shown, to ensure that the experiments were repeatable and statistically sound.

III. RESULTS

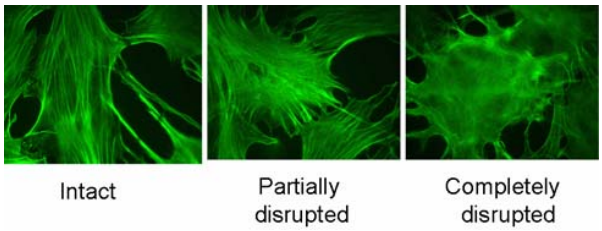

Fig. 1 Images of MLO-A5 cells depicting examples of f-actin cytoskeloton filiments. The left example is of intact f-actin. The middle is of partially disrupted f-actin. The image on the right is of completely disrupted f-atcin. Approximately 400 cells were classified into one of these three categories.

The results from figure 2. show that control cells, which were not loaded, some of the cells exhibited different f-actin states. It is clear that just before beginning of stretch, approximately 75% of the cells have an intact cytoskeleton while 15% and 10% have a partial and completely disrupted cytoskeleton, respectively. When 1% and 3% stretch is applied, the numbers of cells with an intact cytoskeleton are decreased, , while there is a corresponding increase in numbers of cells with a partially disrupted cytoskeleton (Fig. 2a and 2b for 1% and 3% substrate stretch respectively).

With an increase in the magnitude of stretch, a larger number of cells exhibit a partially disrupted cytoskeleton (25% at 1% stretch compared to 48% at 3% stretch). 180 seconds after stretching, the numbers of cells with an intact cytoskeleton reach control levels (i.e. to the 75% level that is shown before stretch was applied). These results indicate that f-actin was disrupted in some cells immediately after stretch. However, over time there were changes in the relative proportions of cells exhibiting different f-actin states. Within 180 seconds after the end of mechanical stimulus, the f-actin states were within control levels. This result suggest that depolymerization of f-actin in cells may depend on the initial stress state of the cell and the magnitude of stretching. Qualitatively, 180 seconds after the end of mechanical stimulus, there was no difference in f-actin organization in control and stretched cells.

Increased disruption of the f-actin fiber bundles were also observed in response to increasing the numbers of loading cycles, increasing frequency, insertion of rest between stretch cycles, as well as by reducing the amount of extracellular calcium.

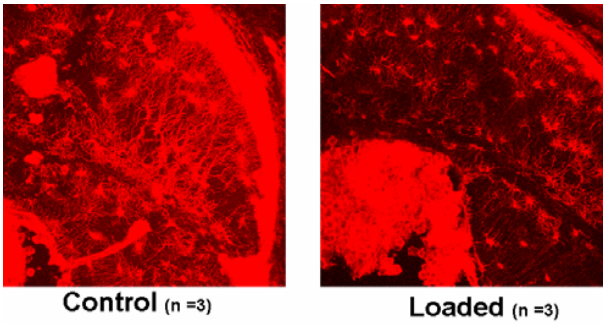

Fig. 2. As the magnitude of stretch was increased from 1% to 3% the so increased the number of cells with a disrupted cell membrane.

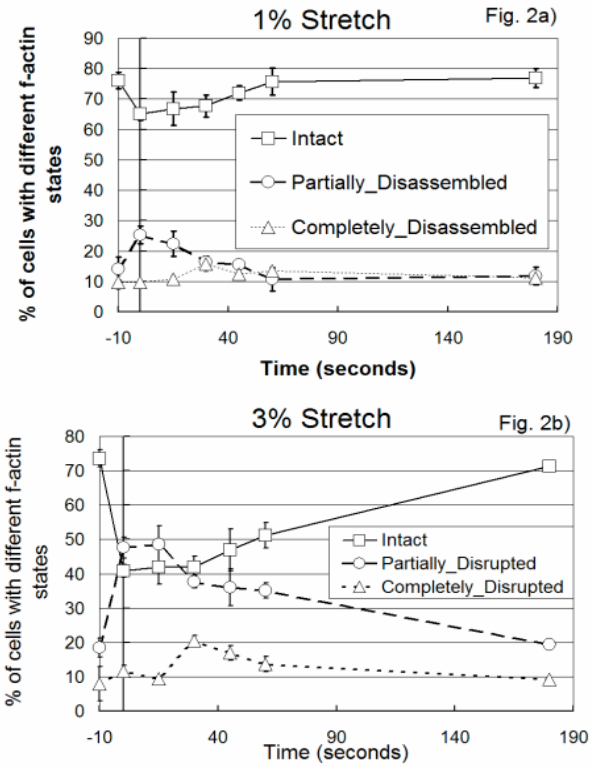

Fig. 3. This figure illustrates the effects of mechanical loading on the forearm of mice. On the left is an on loaded control specimen. On the right is a loaded experiment.

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