Light meets force: Real-time cellular adaptation to mechanical stresses revealed by integrated microscopy

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INTRODUCTION

Mechanical forces directly impact tissue formation and cell function. The structure-function mechanistic-based research represents the new quantitative biology of the 21st century. The need to see structurally the molecular components of the cell and their functionally related spatiotemporal changes under mechanochemical stimuli encouraged the development of enabling technologies that make possible simultaneous mechanical stimulation coupled with optical microscopy of live cells in real time. Thus, the non-traditional integration of available microscopy techniques opens new opportunities for real-time mechanotransduction studies at the single cell level; that is the conversion of mechanical signals into intracellular biochemical signals responsible for coordinating cellular adaptive remodeling necessary to maintain the normal physiological function.

Studying live cells in real time is important because it prevents the possibility that some of the cellular components may be lost or distorted during specimen preparation, always a matter of concern when using the classical methods of fixing and staining cells which offer information of the cellular structural status only at one time point. These studies involve the use of live cells expressing green fluorescent proteins (GFP) or their engineered variants [1, 2]. Thus, genetically expressed GFP-fusion tagged proteins enable fluorescence imaging of dynamic sub-cellular structure remodeling using time-lapse imaging.

Cellular responses to mechanical stresses play an important role in the physiology of many cell types in healthy and diseased states. Physiological adaptations to local forces induce realignment of the cell cytoskeleton and redistribution of cell adhesions, altering the cellular structure-function relationship. The vascular wall is an important example where transduction of mechanical signals along intracellular pathways may alter cell growth, proliferation, adhesion and contraction [3, 4]. Remodeling of the vascular wall is a significant component of the classical progression of disease in atherosclerosis and hypertension, where changes in vascular wall structure substantially impact vascular control and reactivity.

This study investigates real-time cellular adaptation to external tensile stresses that mimic the in-vivo axial stresses which are considered to play an important role in blood vessel homeostasis [5].

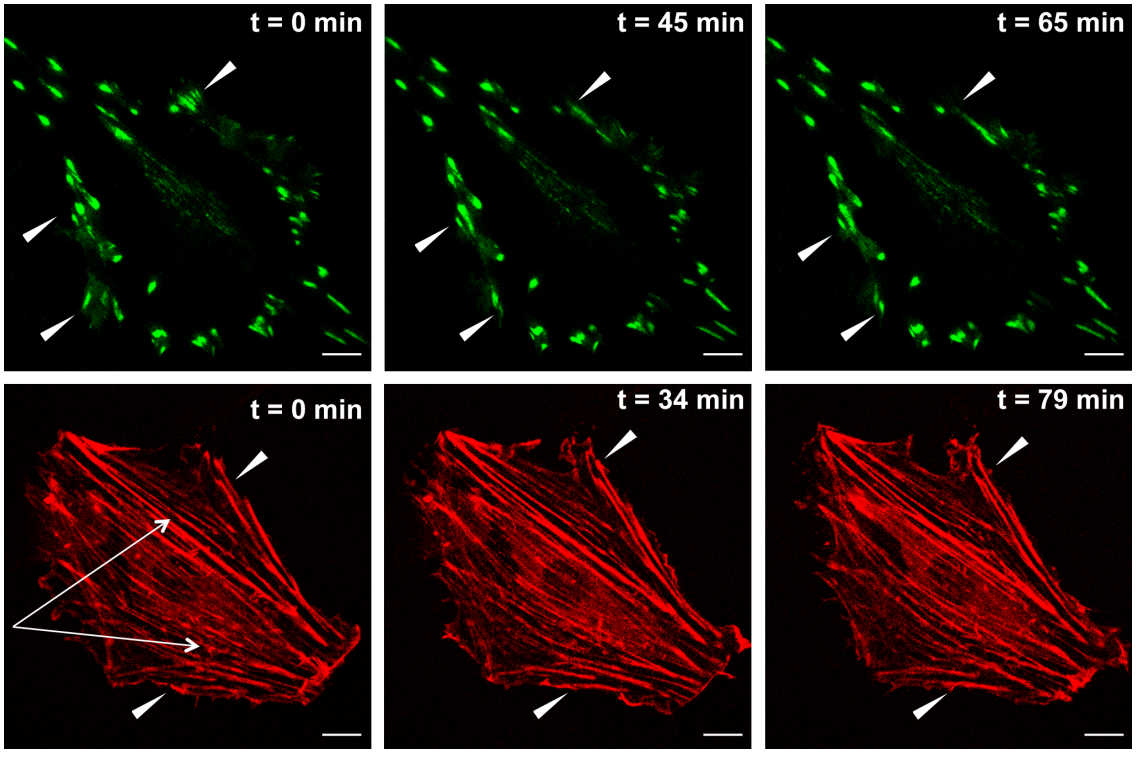

Figure 1
Representative images of vascular smooth muscle cells expressing vinculin-GFP (top row) or actin-mRFP (bottom row) that were mechanically stimulated with a fibronectin-functionalized AFM probe and simultaneously imaged by TIRF or spinning-disk confocal microscopy, respectively. The arrows indicate focal adhesion areas or actin fibers that show remarkable restructuring due to mechanical stimulation. Thus, actin fibers align parallel along the long axis of the cell (arrows), or form bundles at cell edges (arrow heads). Scale bars = 10 µm. This set of images was adapted from [6].

MATERIALS AND METHODS

CELL CULTURE AND FLUORESCENT REPORTERS

Transient transfections of low passage vascular smooth muscle cells (VSMC) in suspension were performed using an Amaxa Biosystems Nucleofector apparatus. Cells were plated for 24 hours on MatTek glass bottom dishes before experiments were performed [6].

VSMC were a gift of Michael Davis, Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO. pcDNA3-EGFP-RhoA-wild type (wt) and mutants (T19N – dominant negative, Q63L – constitutively active) were purchased from Addgene [7]. Actin-mRFP plasmid was a gift of Michael Davidson (Florida State University, Tallahassee, FL), and vinculin-GFP plasmid was a gift of Kenneth Yamada (National Institutes of Health, National Institute of Dental and Craniofacial Research, Bethesda, MD).

INTEGRATED MICROSCOPY TECHNIQUES

A Bruker Bioscope SZ atomic force microscope (AFM) was mounted on top of an inverted Olympus IX-81 optical microscope equipped with a total internal reflection fluorescence (TIRF) attachment. This optical microscope was further combined with a CSU-22 Yokogawa spinning-disk confocal scanning head. Two identical Photometrics QuantEM 512 SC cameras and an Olympus PLAN APO 60x oil 1.45 NA objective lens were used for imaging live cells expressing fluorescent protein constructs.

This integrated microscope system [8] that combines the structural and functional resolving power of the AFM with rapid optical imaging at high spatial and temporal resolution by TIRF and spinning-disk confocal imaging in one instrument represents an important advancement for real-time studies of mechanotransduction-induced dynamics of sub-cellular structures in live cells.

Briefly, AFM [9] uses a nanosensor to sense the physical profile of the cell surface and can directly apply and measure mechanical force with high precision. TIRF and spinning-disk confocal microscopy are camera-based optical imaging techniques. The use of highly sensitive cameras that allow low-level laser excitation are the best for long time-lapse imaging. Electron-multiplied charge-coupled device (EM-CCD) cameras provide high quality images with a good signal-to-noise ratio and require a minimal exposure of cells to light, thus preventing cell photo-toxicity. TIRF microscopy is based on the total internal reflection phenomenon at the cell-coverslip interface, providing high contrast images with a high resolution along the z-axis of any fluorescently labeled molecules in the immediate vicinity of the cell-coverslip interface (< 100 nm depth) [10]. Spinning-disk confocal microscopy [11] is a multilaser beam scanning method that allows three-dimensional optical sectioning of live cells for rapid spatial imaging of cytoskeletal proteins dynamics throughout the cell body.

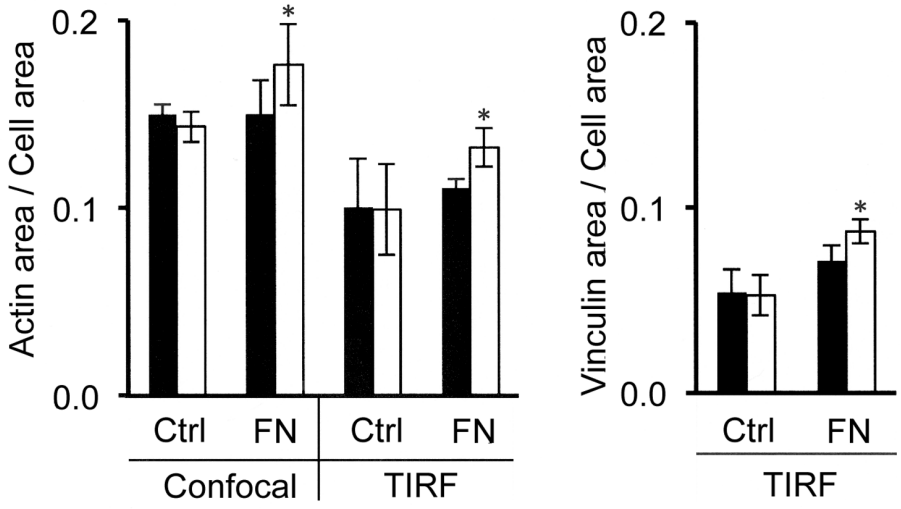

Figure 2
Quantification of relative vinculin and actin protein areas before (black bars) and after (white bars) mechanical stimulation. Confocal images were used to measure actin fibers throughout the cell, while TIRF images were used to measure protein area at the basal cell surface. Data are presented as means ±SEM. Significance was evaluated at $p<0.05$. The graph was redrawn from [6].

MECHANICAL STIMULATION OF LIVE CELLS

Cells were mechanically stimulated by an AFM probe that enables direct application of tensile stress (i.e. vertical pulling away from the cell) and has been described in detail [6, 12]. The AFM probe consisted of a fibronectin (FN) functionalized 2-µm diameter glass bead attached to a silicon nitride cantilever by Novascan Technologies. The AFM probe was kept in contact with the apical cell surface for 20 min to initiate the formation of an adhesion contact, and then very low magnitude forces (<0.4 nN) were applied to further induce focal adhesion protein recruitment to obtain a strong functional focal adhesion (FA). After this priming period, the cell was mechanically stimulated by discrete applications of low (0.5 nN) and high (1 nN) magnitude forces at 3–5 min for 20–25 min each. FN probes remained functionally attached for the duration of the experiment (> 80 min) by engaging endogenous $\alpha_5\beta_1$ integrins, FA proteins and cortical actin, while uncoated probes detached from the cell surface in the priming period.

RESULTS AND DISCUSSION

Cytoskeleton and focal adhesions are dynamic structures that have significant functions in regulating cell migration, proliferation, and survival. Knowing their specific distribution and dynamics provides a better understanding of how cells communicate with the matrix and between themselves. These issues become important for local vessel remodeling in response to altered hemodynamic forces. Focal adhesions function as signaling centers for transduction of mechanical forces between the cytoskeleton and matrix, being critical points for the regulation of actin organization. The efficiency of external force transfer through the cytoskeletal network depends on the pre-existing cytoskeletal tension [13], and has a direct consequence on the ability of the cell to adapt to external mechanical stimuli.

MECHANICAL STIMULATION INDUCES CELLULAR REMODELING

Tensile stress applied to the cell by the FN-functionalized AFM probe induced rearrangement of actin filaments and vinculin recruitment at the basal cell area. Figure 1 shows representative images of VSMC expressing vinculin-GFP or actin-mRFP that were mechanically stimulated, and simultaneously imaged by TIRF or spinning-disk confocal microscopy, respectively. FA rearrangement and actin fibers restructuring are shown by white arrows. These remodeling events were triggered by external tensile stress applied to the cell which in turn elicited the fibronectin-integrin-actin linkage in the mechanotransduction process that induced cellular adaptation to the external mechanical cues. Quantification of actin fibers and vinculin relative area before and after mechanical stimulation for FN-functionalized and uncoated (i.e. control) probes is shown in Figure 2.

CELL ADAPTIVE RESPONSE TO MECHANICAL STIMULATION DEPENDS ON CYTOSKELETAL TENSION

RhoA GTPase is of particular interest as a potential modulator of mechanical signaling, playing an important role in cardiovascular disease as a regulator of cell contraction [14]. Activation of the RhoA pathway in animal models and in human patients has been associated with hypertension, while its inhibition has a protective effect on vascular wall remodeling inducing VSMC relaxation [15].

To determine the effect of pre-existing cytoskeletal tension on cell adaptability to tensile stress, VSMC expressing RhoA-mutants have been used to mimic pathological conditions within the vessel wall. Figure 3 shows representative images of VSMC co-expressing actin-mRFP and RhoA-wt, RhoA-DN (dominant negative) or RhoA-CA-EGFP (constitutively active) before and after mechanical stimulation. As a pre-existent cytoskeletal morphology to the mechanical stimulation (Figure 3A), RhoA-CA induced a significant increase in actin stress fiber formation (Figure 3B), while RhoA inhibition reduced stress fibers spanning across the cell body, with actin fibers present only at the cell edges. Actin fibers remodeling in response to mechanical stimulation is mostly evident in RhoA-CA expressing cells (see white arrow heads).

Overall probe displacement over time has been recorded simultaneously with fluorescence imaging and is shown in Figure 4. AFM tensile stress stimulation at the apical cell surface induced significantly different cell responses that depend on pre-existing cytoskeletal tension. The AFM probe detached from the cells expressing RhoA-DN at the first application of high force (1 nN), because the FA formed around the functionalized probe were weak due to absence of a strong cortical actin network. The displacement over time in this case showed a linear dependence. However, RhoA-CA expressing cells were found to resist the tensile stress by pulling back and exhibited a high reactive response with time. In contrast, cells treated with ML-7, a potent myosin inhibitor, presented an overall dependence that plateaus at high forces due to blocking of the actomyosin apparatus, hence a reduced cellular contractility without actin fibers disassembly.

CONCLUSIONS

Our results suggest that the ability of cells to adapt to external forces depends on the pre-existing cytoskeletal tension which is determined by the actomyosin function. Both actin cytoskeletal network integrity as well as myosin-induced contractility are responsible for regulation of cell adaptive response to microenvironmental mechanical cues.

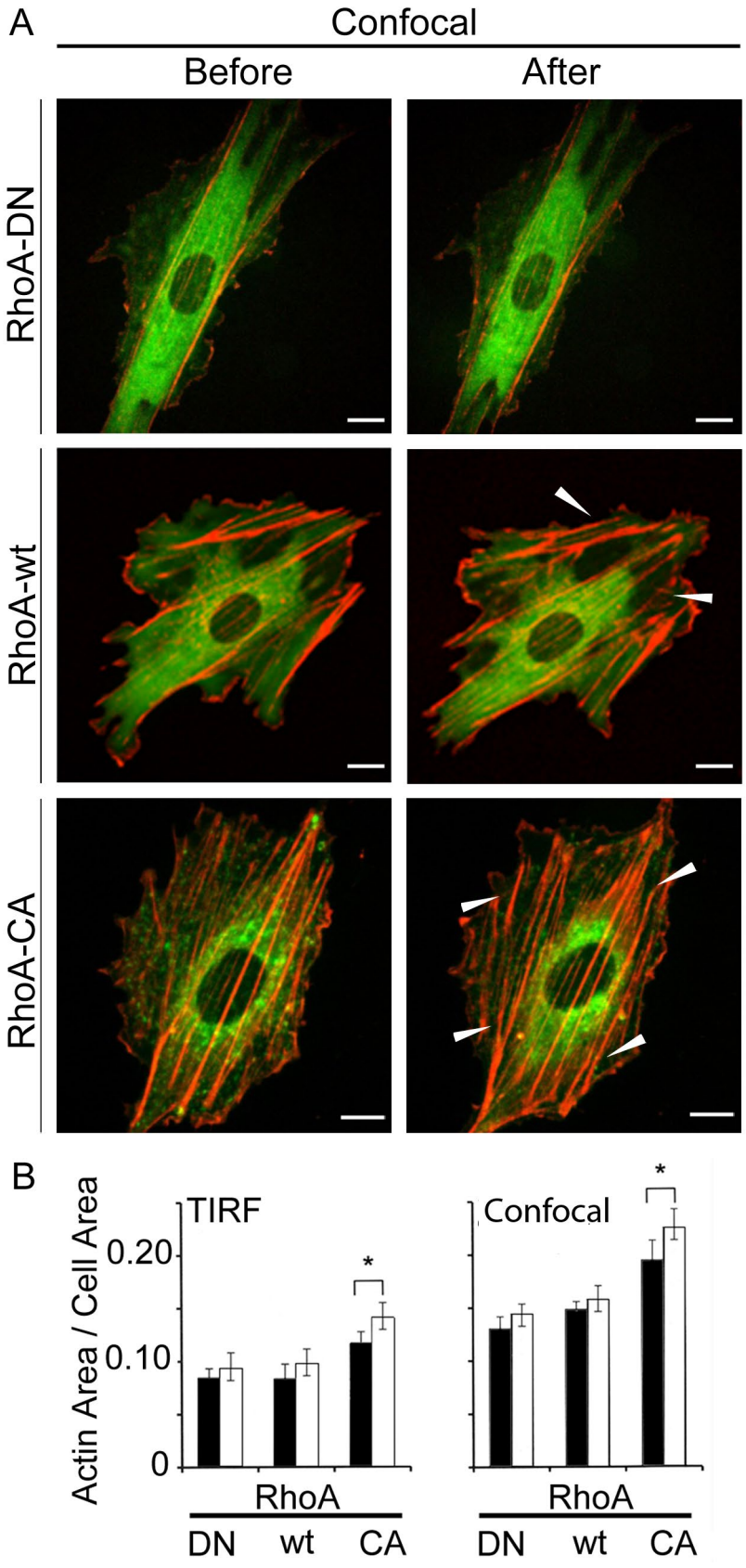

Figure 3
(A) Representative confocal images of the same VSMC co-expressing RhoA-EGFP constructs (green) and actin-mRFP (red) are presented for each treatment. Images were acquired before and after mechanical stimulation experiment. Arrowheads show remodeling of actin fibers. Scale bar is 10 μm. (B) Relative actin area before (black) and after (white) mechanical stimulation is presented as mean ± SEM. Significance was evaluated at $p<0.05$. This image was reproduced from [6].

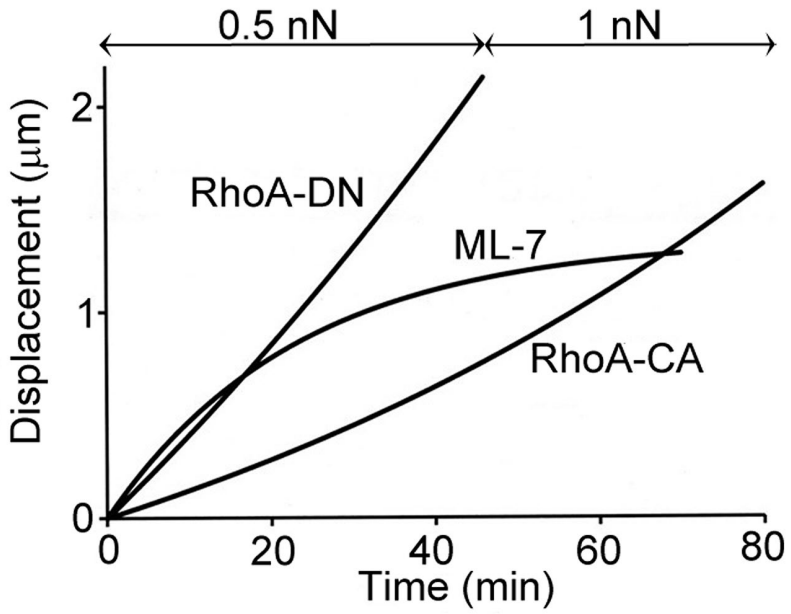

Figure 4
Cytoskeletal tension modulates cellular adaptive response to force. Overall displacements for each treatment over the duration of the mechanical stimulation experiments are shown. This graph was adapted from [6].

By combining mechanical stimulation with simultaneous fluorescence imaging of live VSMC expressing RhoA mutants, we have studied the role of RhoA-induced cytoskeletal tension on the adaptive cellular remodeling to external mechanical signaling. These measurements were enabled by our integrated microscopy system that used a matrix functionalized AFM probe for mechanical stimulation of live cells, coupled with EMCCD camera-based fluorescence imaging using TIRF and spinning-disk microscopy. This experimental approach enabled spatiotemporal quantitative measurements that showed real-time structural and functional dynamical adaptations of the cell in the process of mechanotransduction.

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BIOGRAPHY

Andreea Trache received her PhD in physics from the Institute of Atomic Physics, Romania, and completed her postdoctoral training at Texas A&M Health Science Center, College Station, TX. Currently, she is associate professor in the Department of Medical Physiology at Texas A&M Health Science Center. Her research is focused on the study of molecular mechanisms responsible for altering the intracellular cytoskeletal force balance and the effects of mechanotransduction on focal adhesions and cytoskeletal remodeling.

ABSTRACT

Real-time mechanically induced cellular remodeling is a critical component for understanding cell adaptive response to microenvironmental mechanical cues. Mechanical stimulation of live cells by an atomic force microscope probe combined with simultaneous fluorescence imaging by spinning-disk confocal and total internal reflection fluorescence microscopy enabled visualization and quantification of cytoskeletal structure dynamics in real time. Results provide evidence that cellular adaptive responses to the external forces depend on the pre-existing cytoskeletal tension which is determined by actin cytoskeletal network integrity as well as myosin-induced contractility.
Keywords: mechanical stimulation, fluorescence imaging, RhoA, focal adhesions, actin, vascular smooth muscle

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