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Cyclic Hydraulic Pressure and Fluid Flow Differentially Modulate Cytoskeleton Re-Organization in MC3T3 Osteoblasts

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Abstract

Mechanical loads are essential towards maintaining bone mass and skeletal integrity. Such loads generate various stimuli at the cellular level, including cyclic hydraulic pressure (CHP) and fluid shear stress (FSS). To gain insight into the anabolic responses of osteoblasts to CHP and FSS, we subjected MC3T3-E1 preosteoblasts to either FSS (12 dynes/cm²) or CHP varying from 0 to 68 kPa at 0.5 Hz. As with FSS, CHP produced a significant increase in ATP release over static controls within 5 min of onset. Cell stiffness examined by atomic force microscopy increased after 15 min of either CHP or FSS stimulation, which was attenuated when extracellular ATP was hydrolyzed with apyrase. As previously shown FSS induced polymerization of actins into stress fibers. However, the microtubule network was completely disrupted under FSS. In contrast, CHP appeared to maintain strong microtubule and f-actin networks. The purinergic signaling was found to be involved in the remodeling of f-actin, but not microtubule. Both CHP and FSS applied for 1 hour increased expression of COX-2. These data indicate that, while CHP and FSS produce similar anabolic responses, these stimuli have very different effects on the cytoskeleton remodeling and could contribute to loss of mechanosensitivity with extended loading.

Keywords

mechanotransduction; f-actin; microtubule; ATP release; cell stiffness; COX-2; AFM

INTRODUCTION

Mechanical loading incurred during daily activities is essential towards maintaining the structural integrity of bone and bone mass. The absence of mechanical loads, such as during immobilization or exposure to microgravity, has been shown to induce rapid bone loss.^{1, 2} In contrast, exogenous loading has been shown to increase bone formation, which is well documented at the tissue level.^{3, 4} However, there is a limited understanding regarding the underlying mechanism involved at the cellular level. Gross mechanical loading of the skeleton during locomotion generates a variety of stimuli, including substrate strain, stress, pressure, and interstitial fluid flow that can trigger anabolic responses in osteoblasts.⁴ For example, dynamic loading induces deformation of the extracellular matrix that, in turn, generates cyclic hydraulic pressure (CHP) and interstitial fluid shear stress (FSS) around the cells. Because tissue deformation incurred during in vivo loading is less than 0.3%, FSS is often regarded as

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the primary stimulus for cellular responses associated with bone adaptation.^{5–8} The short and long-term influence of FSS mediated by specific signaling pathways in osteoblasts has been extensively studied in-vitro using uni-directional, or oscillating flow patterns.^{5, 7}

A time course of events that occur in osteoblasts in response to FSS is beginning to emerge. Within seconds of the onset of FSS, osteoblasts respond with a rapid increase in intracellular calcium concentration that is dependent on both extracellular calcium influx and calcium release from intracellular stores.⁹ Extracellular calcium entry is mediated through the activation of both mechanosensitive cation-selective channels (MSCC) and L-type voltage sensitive calcium channels in osteoblasts.^{10, 11} This calcium entry leads to vesicular release of ATP within 1 min of the onset of FSS.¹² ATP, in turn, binds to purinergic (P2) receptors on the plasma membrane that can mediate the release of intracellular calcium stores through stimulation of phospholipase-C and D-myo-inositol 1,4,5-triphosphate (PLC/IP₃).^{13, 14} This increase in intracellular [Ca²⁺] influx can also modulate cytoskeleton polymerization that occurs within 30 min of application of FSS.^{13, 15} Polymerization of the cytoskeleton has been shown to alter cell stiffness,^{16, 17} which may regulate the sensitivity of bone cells to subsequent mechanical stimulation, including the activity of the MSCC.^{17, 18} Ultimately, mechanical stimulation of osteoblasts leads to altered gene expression through activation of transcription factors such as *c-fos* and NFκB.^{19, 20} These changes in gene expression, in particular cyclooxygenase-2 (COX-2), lead to increase bone formation and counteract disuse bone loss in vivo.^{2, 21}

Although these in vitro fluid chamber studies successfully mimic in-vivo flow conditions experienced by bone cells, they fail to replicate many of the stimuli occurred in bone during locomotion. One possible stimulus is the hydraulic pressure that may influence bone adaptation. The pressures used to drive fluid flows in these flow chamber systems is usually on the order of 1–3 kPa due to the low resistance of the chamber. A much higher flow resistance exists in the porous bone matrix due to its extremely low permeability,⁶ resulting in a transient hydraulic pressure as high as 2 MPa inside bone under physiological loading conditions.²² Although accessibility issues limit direct measurements of the pressure perceived by osteoblasts and osteocytes, the pressure within the marrow cavities of turkey ulnae was found to be 8.6 kPa for a step compression of 600 microstrain.²³ The intramedullary pressure varied with loading magnitude and frequency, and could reach a magnitude as high as 30–40 kPa (Qin Y-X, personal communications). While several reports suggest the importance of CHP in bone adaptation, the responses of bone cells to CHP is less well understood. At the tissue level, increased intramedullary pressure induced by venous stasis or exogenously applied pressurization was associated with increased bone formation.^{24, 25} At the cellular level, pressure has been shown to increase intracellular [Ca²⁺]_i, and alter gene expression related to osteogenesis.^{9, 26–30} In contrast to the numerous studies involving FSS, there is a limited understanding regarding the early responses of osteoblasts to CHP.

To evaluate differences in osteoblastic responses to FSS and CHP, we stimulated MC3T3-E1 osteoblast-like cells with FSS or CHP and measured short-term (e.g., ATP release), intermediate (e.g., cell stiffness), and long term responses (e.g., COX-2, cytoskeleton remodeling) to these stimuli. We postulated that CHP stimulates an anabolic response similar that that of FSS, but that CHP mediates these effects through different signaling pathways. We found that both FSS and CHP increased ATP release, cell stiffening and COX-2 expression in a similar manner. However, FSS and CHP differentially regulated the cytoskeletal re-organization in osteoblasts.

METHODS

Cell Culture

MC3T3-E1 osteoblast-like cells (Clone 14, ATCC, Bethesda, MD) were cultured in α -MEM containing 10% FBS (Gibco, New York, NY), 100 U/ml penicillin, 100 μ g/ml streptomycin and 26 mM NaHCO₃. Cells were maintained within humidified incubators at 37° C with 5% CO₂/95% air. Cells were seeded on glass slides coated with rat-tail type I collagen (50 μ g/ml, BD, Franklin Lakes, NJ). Once cells reached 75% confluence, they were serum starved for 24 hours prior to testing with serum free medium containing 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 26 mM NaHCO₃. All the compounds, if not stated otherwise, were purchased from Fisher Scientific (Pittsburgh, PA).

In-Vitro CHP and FF Stimuli

A custom-built pressure chamber was used to expose cells to CHP. The pressure system consisted of a linear syringe pump (PHD 22/2000, Harvard Apparatus) with a 150 ml syringe connected to the inlet of the chamber (Fig. 1A). The glass slide seeded with cells was positioned inside the chamber, resting on the base of the chamber to avoid bending of the glass slide. To generate hydraulic pressure, the syringe plunger was driven by the pump and pressed against a flexible membrane fixed inside the syringe. The flexible membrane isolated 25 ml of serum free medium that filled inside the syringe, connecting tubing and pressure chamber. Minimal deflection of the membrane (< 2 mm) was required to pressurize the fluid chamber and the intra-chamber pressure was monitored using a digital monometer (DPG1000B, Omega Inc., Stamford, CT). Due to the small deflection of the membrane and relatively large fluid volume inside the whole system, fluid motion over the cells was negligible. Cells were subjected to a hydraulic pressure varying from 0 to 68 kPa at a rate of 0.5 cycle per sec (Hz), which were within the range of 8 kPa-3 MPa at 0.3–20 Hz used in previous studies.^{9, 22, 25, 26, 28, 29, 31, 32}

Cells were also subjected to FSS using a parallel plate flow system that generated a laminar uni-directional flow across the cells as described by previous studies.⁵ The flow system was maintained at 37°C, and was filled with 25ml of serum free medium that was aerated with 5% CO₂/95% air. The laminar flow rate was adjusted to generate a wall shear stress of 12 dynes/cm² under a constant pressure difference of ~2 kPa.

Control experiments were performed separately for each stimulus by placing the cells in either the pressure chamber or the parallel plate chamber but without applying the corresponding stimulus. The volume and gas partial pressures in the media and duration of the control experiments remained the same as the corresponding FSS and CHP experiments. Since no difference was found between these control experiments in the outcome measures described below, the two types of control experiments were pooled and results were reported under a common “static control” group.

Outcome Measures

The responses of MC3T3 cells to either CHP or FF were evaluated at various time points (5, 15, or 60 min) after the onset of stimuli. The measured outcomes detailed in the following sections include: ATP release, cellular stiffness, COX-2 expression, and cytoskeleton organization. Each experiment was repeated a minimum of three times.

ATP Assay—MC3T3-E1 cells were exposed to CHP or FSS for 5 min, following which 15 ml of the culture media was taken and stored at –80°C. Once an appropriate number of samples had been obtained, the released ATP concentration was measured using a bioluminescence assay kit (ATP Bioluminescence Assay kit HS II; Roche, Indianapolis, IN). Light emitted as

a result of the reaction between D-luciferin and luciferase was detected using a 96 well micro-injector plate reader (POLARstar OPTIMA, BMG LABTECH GmbH). To normalize the measured ATP release to total cell protein, the MC3T3-E1 cells were washed with PBS immediately after exposure to CHP or FSS, and then incubated at -20°C with 80 μl of lysis buffer (5 mM HEPES, 150 mM NaCl, 26% glycerol, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The protein samples were stored at -80°C for further analysis. The protein concentration of each sample was determined using a BCA assay (BCA Protein Assay Kit, Pierce) and the 96-well micro-injector plate reader.

COX-2 Western Blot—To establish that CHP can induce an anabolic response on osteoblasts as does FSS, COX-2 expression was evaluated using western blot analysis. Our previous studies have shown FSS can induce a significant increase in COX-2 expression as early as one hour after the onset of flow, cells in this study were exposed to one hour of either FSS or CHP. 13, 15, 19 Cells exposed to either FSS or CHP, along with static controls, were first washed with PBS and then incubated at -20°C in 80 μl of lysis buffer. Lysate samples were electrophoretically transferred to a nitrocellulose membrane and then blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.05% Tween-20 (TBST) for 1 hour. The membrane was incubated overnight at 4°C with 1 $\mu\text{g}/\text{ml}$ rabbit antibodies to COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed with TBST and incubated with IgG anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. Immunodetection of COX-2 was achieved using an Enhanced Chemiluminescence kit (NEN Life Science Products, Boston, MA). To establish a loading control, the membrane was stripped and incubated with 1 $\mu\text{g}/\text{ml}$ rabbit antibodies for vinculin overnight at 4°C and then incubated with the same secondary antibody for immunodetection as described for COX-2. Vinculin is a focal adhesion protein that has been shown to remain unchanged in response to mechanical stimuli.^{15, 19} Densitometry software (ImageJ, National Institute of Health) was used to determine the blot area and intensity of COX-2 and vinculin. The expression of COX-2 was normalized with the expression of vinculin and this normalized expression of COX2 was compared among FSS, CHP, and static controls.

Cellular Stiffness—After cells were exposed to 15 min of either FSS, CHP, or static controls, the apparent elasticity was measured using an Atomic Force Microscope (AFM) (BioScope II, Veeco Inc.) mounted on an inverted optical microscope. For each group, five to seven cells were randomly selected for measurement within 60 min as described below. Soft microlever probes (MLCT-AUNM, Veeco Inc.) with a conical tip and a spring constant of 0.01 N/m were calibrated using a thermal fluctuation method in fluid. Individual cells with normal morphology were identified under the optical microscope, and positioned under the probe. An area of 30 $\mu\text{m} \times 30 \mu\text{m}$ was first scanned at a speed of $\sim 3 \mu\text{m}/\text{sec}$ to generate a topographic map of the individual cell. Seven to ten points were selected over the cell body at the nuclear and perinuclear regions for indentation measurements. The cell peripheral region was avoided due to its relatively thinner cell height, where the rigid substrate may cause overestimation of the apparent stiffness.³³ At each point, the cantilever tip approached the cell at a speed of $\sim 2.5 \mu\text{m}/\text{sec}$ until a force of 100 pN was reached, which typically resulted in an indentation depth of $< 70 \text{ nm}$ (10% of the cell height). The time for each indentation cycles (loading, unloading, and resting) took approximately 1–2 sec. Although the force-deflection curves show the typical hysteresis between loading and unloading, we used the unloading curve to derive the elastic modulus and thus maintained consistency in comparing the values for different groups. The elastic modulus at each point was estimated from the recorded force-deflection curve using a Hertz based model given below:^{34, 35}

$$E = \frac{2F(1 - \nu^2)}{\pi \delta^2 \tan(\varphi)} \quad (\text{Eq. 1})$$

where E is the apparent stiffness, F is the cantilever force measured by the AFM, ν is the Poisson ratio of the cytoplasm ($\nu = 0.4$),³⁶ φ is the opening angle of the conical cantilever tip ($\varphi = 35^\circ$), and δ is the indentation depth, which was calculated by subtracting the cantilever deflection from the piezo displacement of the probe. The averaged apparent stiffness from these points was obtained for each cell, and the modulus for the cells stimulated with FSS, CHP, or static control was averaged across 3 to 4 repeated experiments. The fold increase in apparent cell stiffness for each stimulus relative to static controls was reported as mean $\pm$ SEM.

Cytoskeleton Immunostaining—The f-actin and microtubules, the two main components of cytoskeleton, were visualized using immunostaining to evaluate the influence FSS or CHP on the cytoskeleton organization compared to static controls. Previous studies have shown that one hour of FSS can cause significant changes in f-actin alignment.^{13, 15, 18} Thus, the cells were stimulated for 1 hour with either FSS or CHP. The stimulated cells and their static controls were then fixed with 0.25% glutaraldehyde and permeabilized with 0.1% Triton X-100 in PHEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 2 mM MgCl, pH 6.9, warmed to 37°C) for 30 min. The PHEM buffer ensured that microtubules would remain intact during the staining process.³⁷ Following fixation, cells were quenched in 2 μ g/ml of sodium borohydride for 15 min to reduce the auto-fluorescence of glutaraldehyde. Nonspecific binding was reduced with 10% BSA (Gibco, New York, NY) treatment for 1 hour. Microtubules were first labeled with alpha-tubulin antibody (AB Cam, Cambridge, MA) for 3 hours, and then with Cy-3 secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 1 hour. F-actin was then labeled green by incubating the cells with Alexa Fluor 488 phalloidin conjugate (Invitrogen) for 1 hour. This protocol has been used previously to successfully avoid disruption of microtubules.^{37, 38} After mounting the slides with polyvinyl alcohol mounting medium (Fluka-BioChemika), the f-actin and microtubule structures were imaged under dual excitations (488 nm for f-actin labeling and 561 nm for microtubule labeling) using confocal microscopy (LSM 510, Zeiss, Germany) with a 100 $\times$ oil immersion lens at the mid-plane of the cells.

Purinergic Signaling in Mechanotransduction—We have previously demonstrated that re-organization of f-actin is specifically dependent upon PLC-mediated intracellular Ca^{2+} release.¹³ However, the PLC pathway is typically activated through G-proteins. We postulated that this activation is mediated through purinergic receptors. To demonstrate that ATP signaling not only stimulates f-actin polymerization during mechanical stimulation but also evaluate its influence on the microtubules, static control cells were exposed to exogenous ATP (100 μ M, Sigma) in serum free medium for 30 min. To inhibit ATP activation of purinergic receptors, we used apyrase (5 U/ml, Sigma) in culture media to hydrolyze extracellular ATP during either FSS or CHP stimulation. Cell stiffness, cytoskeletal organization, and COX-2 production were evaluated in response to exogenous ATP addition and to FSS or CHP in the presence of apyrase in the medium.

Statistical Analysis

One-way ANOVA was used to detect differences among experimental groups for various mechanical stimuli (CHP, FSS, and static controls) or various treatment groups. (ATP, apyrase, and controls). Statistical significance was defined with a $p < 0.05$ and a post hoc Turkey-Kramer test was used to detect significant difference among groups.

RESULTS

Effects of FSS and CHP on anabolic responses in MC3T3-E1 cells

ATP release, and its sequential binding to purinergic receptors, has been shown to have significant anabolic effects in osteoblasts^{39, 40}. In this study, application of either FSS or CHP induced a significant increase in ATP release from MC3T3-E1 osteoblasts within 5 min after the onset of the mechanical stimulus compared with static controls (Fig. 2A). FSS produced a larger increase in ATP release (~7-fold) than CHP (~4-fold), but the fold increase was not significant between the two stimuli (Fig. 2A).

Mechanically-induced bone formation is dependent on the synthesis and release of prostaglandins from osteoblasts.^{2, 21} Increase in the production of the inducible isoform of cyclooxygenase, COX-2, is central to this increase in bone formation and prevention of disuse bone loss.^{2, 21} Both FSS and CHP increased COX-2 protein levels after one hour of the onset of the mechanical stimulation, with FSS increasing COX-2 ~6-fold over static controls versus ~3-fold increase observed with CHP (Fig. 2B). The production of COX-2 in response to both FSS and CHP were statistically greater than that of static controls, but no significance was detected between FSS and CHP groups (Fig. 2B).

Effects of FSS, CHP, and purinergic signaling on Cell Stiffness

We predicted that cells respond to a mechanical stimulus by significantly altering their own mechanical property. To determine if either FSS or CHP changed the cytomechanics of the MC3T3-E1 cells, we measured the apparent stiffness of static and loaded cells using AFM. On average, static MC3T3-E1 cells were found to have an apparent stiffness of 1.7 ± 0.3 kPa with a coefficient of variance of 23%. The average stiffness was increased approximately 6-fold and 4-fold to 6.49 ± 1.3 kPa, and 4.1 ± 0.6 kPa in response to FSS and CHP, respectively (Fig. 3). The apparent cell stiffness in response to either FSS or CHP was significantly greater than static controls, but no significant difference was detected between the FSS and CHP groups.

As we and others have shown previously, ATP release from osteoblasts induces numerous responses within the cell through activation of purinergic receptors.^{13, 14, 39} To determine if purinergic signaling was involved in the changes in cell stiffness, we treated static MC3T3-E1 cells with exogenous ATP (100 μ M) or added apyrase (5 U/ml) to loaded cells to hydrolyze released ATP. We found that addition of exogenous ATP to static cells induced a significant 4.0 ± 0.5 fold increase in cell stiffness compared to static controls (Fig. 3). This ATP-induced cell stiffening in static cells was comparable with that seen in cells subjected to either FSS or CHP. The cell stiffening induced by FSS or CHP was significantly attenuated in the presence of apyrase to nearly the same level of static control (Fig. 3).

Effects of FSS, CHP, and purinergic signaling on actin cytoskeletal re-organization

As we have previously reported, FSS induces a dramatic increase in actin cytoskeletal polymerization in osteoblasts.¹⁵ To determine if CHP produced a similar response, we compared the changes in f-actin polymerization in cells subjected to CHP with those subjected to FSS. Differential responses to FSS and CHP in f-actin reorganization were found after one hour (Figs. 4A–C). Static controls exhibited random organization of f-actin as shown in the diffuse staining concentrated along the peripheral cytoplasm and few actin stress fibers (Fig. 4A). As expected, FSS increases f-actin stress fiber formation with distinct alignment after 1 hour (Fig. 4B). Under CHP stimulus, the f-actin did not appear to be significantly altered in distribution, but the staining intensity appeared to be stronger and less diffuse compared to the static control (Fig. 4C). Similar changes in cytoskeleton organization were observed when cells were subjected to 15 min of stimuli and 45 min of post-incubation (data not shown). Thus, it

would appear that 15 min of mechanical stimuli was enough to trigger actin cytoskeletal remodeling.

To determine if purinergic signaling was involved in the changes in actin cytoskeletal organization, exogenous ATP was added to static cells (positive control) or apyrase was added during application of FSS or CHP (Figs. 4D–F). In parallel with changes in cell stiffness (Fig. 3), the addition of exogenous ATP to static cells induced considerable re-organization of f-actin and stress fiber formation (Fig. 4D), although the orientation of these stress fibers were not aligned to any dominant direction, unlike the flow-aligned stress fibers under FSS (Fig. 4B). The addition of apyrase during FSS inhibited re-organization of f-actin (Fig. 4E), but had little effect on actin structure in cells subjected to CHP (Fig. 4F).

Effects of FSS, CHP, and purinergic signaling on microtubule re-organization

To determine if FSS and CHP altered microtubule structure, we stained α -tubulin after 15 min exposure. Static cells exhibited classic microtubule structure, with microtubules emanating from the microtubule organizational center (MTOC) near the nucleus (Fig. 5A). Surprisingly, when MC3T3-E1 cells were subjected to FSS, microtubule staining was totally eliminated after 1 hour of stimulation except for the primary cilia (Fig. 5B). In contrast, CHP's effects were more subtle, and need to be quantitatively analyzed to detect the differences. It appeared that microtubule staining was enhanced and more dispersed throughout the cytoplasm (Fig. 5C).

Unlike its effect on the actin cytoskeleton, ATP treatment to static cells did not alter the microtubule organization although the microtubule intensity was increased (Figs. 5D). The addition of apyrase did not block the disruption of the microtubule network induced by FSS (Fig. 5E), and appeared to have little effect on the microtubule distribution and slightly decreased the microtubule staining comparable to that of the static controls (Fig. 5F).

DISCUSSION

Mechanical loading experienced by bone during physical activities generates various forms of mechanical stimuli, which are believed to play a significant role in osteogenesis and mechanotransduction.^{4, 41} These cellular stimuli include, but not limited to, strain, stress, pressure, interstitial fluid flow, and hydraulic pressure.^{4, 41} To investigate the cellular responses and mechanotransduction pathways associated with these stimuli, various in-vitro systems have been used to mimic the in-vivo conditions, such as FSS chambers,^{5, 7} substrate stretching,⁴² cell poking or aspiration,⁴³ and hydraulic pressure chambers.^{9, 26, 31} Although the cellular responses to FSS and stretching are relatively well established, CHP receives limited attention so far. Results from the few previous studies demonstrate the long-term effects of CHP on bone mineralization and up-regulation of osteogenic genes in bone cells.^{26, 28} The early responses induced by CHP, however, are poorly understood. This is the first study comparing differences between FSS and CHP induced signal pathways within MC3T3 osteoblasts that occur early during the osteogenic responses.

The CHP regimen used in this study (68 kPa at 0.5Hz) was chosen based on previous in-vitro studies, where the peak hydraulic pressure was varied in a big range such as 8.6 kPa,²³ 13 kPa, 28–40 kPa,³¹ 68 kPa,⁹ and up to 3 MPa.^{29, 32} Several studies have attempted to measure the intramedullary pressure within various animal models.^{23, 44} Intramedullary pressure oscillation was found to be ~1.3 kPa in mice during normal ambulation.⁴⁴ Under a step compression of 600 microstrains, the intramedullary pressure recorded in turkey ulna was increased to ~8.6 kPa,²³ and could reach a magnitude as high as 30–40 kPa by increasing the loading magnitude and frequency (Qin Y-X, personal communication). The load-induced fluid pressure inside the porous bone matrix has not been measured due to accessibility difficulty. Based on theoretical models, fluid pressure within the Haversian canals where the osteoblasts

line on the surface was found to be 19% of the applied stress, i.e., the local pressure could be over 3.4 MPa for a 18 MPa stress.²² Due to the fast relaxation time of pressure within the vascular regions of bone,²² the hydraulic pressure applied in this study was much lower (68 kPa). The frequency of the CHP stimuli (0.5 Hz) was chosen because this is the approximately the stride frequency for human walking.³⁰

Our study showed that a CHP of 68 kPa at 0.5 Hz induced similar anabolic responses within MC3T3-E1 osteoblasts in comparison to FSS of 12 dynes/cm². Both stimuli produce comparable increases in ATP release, cell stiffening, and COX-2 expression relative to static control, which were examined at 5, 15, and 60 min after onset of stimulation (Figs. 2, 3). The increase in the release of ATP and production of COX-2 induced by FSS was in agreement with previous studies.^{12, 13, 19, 40} Previous studies have found that intermittent hydrostatic pressure induces various changes in cells and cultured bone organs such as actin mRNA expression,²⁶ transforming growth factor-beta,²⁷ collagen,²⁹ prostaglandin E,^{45, 49} *c-fos* mRNA expression,⁴⁶ and osteopontin mRNA.²⁸ In agreement with those previous studies, our results showed MC3T3 osteoblasts are responsive to CHP as shown in the observed increase in COX-2 expression (Fig. 2).

Despite similarities in ATP release, changes in cell stiffness, and expression of COX-2, FSS and CHP altered cytoskeleton organization in significantly different ways. As expected, FSS caused an increase in parallel alignment of the f-actin and formation of stress fibers.^{13, 15} CHP also induced moderate changes in f-actin organization shown as decreased diffuse staining and increased fiber staining intensity compared to static controls. Previous studies have shown that chondrocytes and endothelial cells exhibit an increase in f-actin stress fiber formation in response to cyclic pressure.⁴⁷ In agreement with a previous study,¹⁶ our AFM indentation studies suggest that f-actin stress fibers, but not the microtubules, contributed to the cell stiffening, because disruption of f-actin, but not microtubules, abolished the cell stiffening during FSS and CHP (Fig. 3). Furthermore, we clearly demonstrated that the purinergic ATP signaling was involved in the remodeling of f-actin in response to FSS and CHP, which was compatible with previous studies.¹³ However, purinergic ATP signaling appeared not to be involved in microtubule remodeling as the apyrase treatment did not prevent the disruption of the microtubules under FSS.

The most surprising result in this study was that the microtubule network was completely disrupted in response to one hour of FSS, where the only microtubule structure visible was the primary cilia. While this would indicate that our staining protocol was working, we can only speculate that the microtubules depolymerized in response to FSS, and the monomers extracted through the large pores created in the membrane during the permeation protocol using Triton X-100, which may explain the absence of single alpha monomers in our staining (Fig. 5). This result appeared to be contradictory to previous studies by us and others, where microtubules did not undergo significant alterations in MC3T3-E1 osteoblasts subjected to a similar magnitude and duration of FSS.^{19, 48} However, these earlier studies used fibronectin-coated glass as opposed to type-1 collagen used in the present study. This different, albeit more physiologic, matrix protein may contribute to the differential responses we observed in microtubule organization. Different substrate coatings have been found to vary the pre-stress of f-actin and cell stiffness due to the modulation of focal adhesion complex.¹⁶ Similar systemic studies are needed to elucidate whether and how substrates modulate microtubule's re-organization in response to FSS and CHP.

In contrast to FSS, Our results demonstrated that the microtubules could maintain the structural integrity under a 68 kPa hydraulic pressure. The microtubules expanded throughout the cytoplasm, whereas microtubules within static controls were predominately located in the perinuclear region. Another study showed that osteoblasts maintained a strong microtubule

network under 50g hypergravity that induced ~ 6 kPa hydraulic pressure over the cells.⁴⁹ Besides sustaining these uniform normal stresses applied over the entire cells, microtubules have been found to maintain its integrity under short-term high local compressive strains (up to 1%) during AFM indentation, cell poking and aspiration studies.^{17, 43} However, it remains a puzzle why microtubules that appear to be strong enough to sustain normal stresses up to 67 kPa during CHP, could be totally disrupted within 15 to 60 min under a FSS that is four-orders smaller in magnitude (12 dynes/cm², 1.2 Pa). Similar disruption of microtubules was reported for primary osteoblasts under dynamic substrate stretching (1.3% at 0.25 Hz).⁵⁰ These experimental findings suggest that the microtubules in osteoblasts respond to various modes of load (normal vs. shear) quite differently.

We speculate that due to its inhomogeneous structure microtubule network may undergo dramatic different local deformations depending on the mode (shear vs. normal stress) and the magnitude of stimulus. Although the average deformation of the microtubule network is expected to be small (e.g., 0.04% volume change for a 10 kPa hydraulic pressure),³⁰ the local deformations experienced by microtubules can be much higher at certain regions, and thus, trigger the remodeling of the microtubule as shown in increased distribution beyond the perinuclear regions. Obviously these local deformations are not deleteriously high so that the overall integrity of microtubules is maintained CHP (Fig. 5). In contrast, fluid shear has been shown to induce large displacements of cytoskeleton and mitochondrial using real time imaging approaches.^{51–53} These large displacements and structural changes under FSS or stretching may cause the release and activation of microtubule associated proteins, which, in turn, influence the kinematics of microtubule polymerization and depolymerization and lead to the disruption of the microtubules.³⁰

The present studies provide insights into the mechanotransduction mechanisms in osteoblasts. The current consensus is that cytoskeleton plays an important role of transferring extracellular stimuli to a distant location inside cells, although the interactions between different cytoskeleton components during this process is not well understood.^{19, 54} A strong support to this concept comes from a recent study, where rapid activation of Src (within 0.3 sec) was found in remote cytoplasmic sites (20 μ m away) when a living cell was locally deformed with 1.8 Pa using a magnetic bead.⁵⁵ The discrete activation of Src was co-localized with microtubules that underwent significant deformations.⁵⁵ Our study provides additional evidence that different modes of mechanical stimuli (FSS and CHP) elicit dramatic different responses of cytoskeleton while result in similar anabolic responses (e.g., increased COX-2 expression). This finding suggests that multiple mechanotransduction pathways must exist and a living cell usually integrates signals from these pathways to produce a cohesive response in vivo.

In conclusion, we demonstrate that cyclic hydraulic pressure induced similar anabolic responses in the osteoblast as FSS. Furthermore, the change in cell stiffness and re-organization of f-actin was dependent upon load induced ATP release. However, a significant loss of microtubules occurred in response to FSS, while CHP induced an enhanced organization of the microtubule network. These data would suggest that these two types of stimuli alter cytoskeletal organization through separate pathways. Interestingly, while most of the load induced modifications of the cytoskeletal organization was mediated through purinergic signaling, disruption of the microtubules was independent of the release of ATP. We speculate that depolymerization of the microtubules in response to FSS is mediated with activation of the microtubule associated proteins, which needs further investigations.

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Fig. 1.

[A] Schematic of custom-built pressure chamber. The collagen-I coated glass slide seeded with MC3T3 cells was secured to the base of the pressure chamber. The chamber and the connecting tubing and syringe were filled with serum-free medium (~25 mL). A Harvard pump drove the syringe plunger to cyclically deform the flexible membrane inserted within the syringe, generating a cyclic hydraulic pressure (CHP) in the sealed chamber. **[B]** The CHP regimen used in this study consisted of a triangle profile, with a peak pressure of 510 mmHg (68 kPa) at a frequency of 0.5 cycles per sec.

Fig. 2.

Both FSS and CHP induced significant increase in ATP release and COX-2 expression at 5 min and 60 min in MC3T3 osteoblasts. **[A]** FSS induced a greater, but not significant, increase in ATP release over static controls (7-fold), compared to the 4-fold increase induced by CHP. **[B]** FSS and CHP induced similar changes in COX-2, which was increased 5.5 and 3.5 fold over static controls, respectively. No significant difference was found between FSS and CHP groups in ATP release and COX-2 expression ($n = 3$). (* indicates $p < 0.05$ compared to static controls)

Fig. 3.

Early ATP release was involved in MC3T3 cells stiffening under FSS and CHP. The addition of exogenous ATP (100 μ M) to static cells increased the overall cell stiffness by 4-fold, while a comparable increase was found after 15 min of FSS (6-fold) and CHP (4-fold). The FSS- and CHP-induced cell stiffening was almost entirely abolished and the cell stiffness was reduced to that of static control when ATP was hydrolyzed with apyrase. (* indicates $p < 0.05$ compared to static control; ** indicates $p < 0.05$ compared to both static control and apyrase treatment)

Fig. 4.

Both FSS and CHP stimulated significant re-organization of the f-actin network via ATP release. **[A]** Static controls demonstrated a random network of f-actin in the cell periphery and few stress fibers. **[B]** Application of FSS for 60 min stimulated f-actin stress fiber formation and parallel alignment in the direction of flow (left to right). **[C]** Application of CHP for 60 min stimulated an increase in f-actin stress fiber formation as well. **[D]** ATP treatment (100 μM) of static cells increased f-actin formation, similar to those seen under FSS and CHP (**B–C**). **[E and F]** The addition of apyrase that hydrolyzed ATP released by the cells under either FSS or CHP inhibited f-actin re-organization and stress fiber formation. Bar = 20 μm.

Fig. 5.

FSS and CHP induced distinct re-organization of microtubules in MC3T3 cells. **[A]** Static controls exhibited a radial network of microtubules concentrated close to the nuclei. **[B]** Application of FSS for 60 min disrupted the microtubule network except for primary cilia (indicated by arrows). **[C]** Application of CHP for 60 min appeared to enhance the microtubule network as seen in the increased intensity and more spreading of microtubules beyond the peri-nuclear region. **[D]** ATP treatment (100 μ M) of static cells appeared to increase the intensity of the microtubules. **[E]** The addition of apyrase (5 U/mL) did not prevent the disruption of the microtubules induced by FSS. **[F]** The addition of apyrase (5 U/mL) during CHP did not significantly alter the distribution of the microtubules but their staining intensity was reduced to the static level. Bar = 20 μ m.