

Calcium regulates the PI3K-Akt pathway in stretched osteoblasts

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Abstract Mechanical loading plays a vital role in maintaining bone architecture. The process by which osteoblasts convert mechanical signals into biochemical responses leading to bone remodeling is not fully understood. The earliest cellular response detected in mechanically stimulated osteoblasts is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). In this study, we used the clonal mouse osteoblast cell line MC3T3-E1 to show that uniaxial cyclic stretch induces: (1) an immediate increase in $[Ca^{2+}]_i$, and (2) the phosphorylation of critical osteoblast proteins that are implicated in cell proliferation, gene regulation, and cell survival. Our data suggest that cyclic stretch activates the phosphoinositide 3-kinase (PI3K) pathway including: PI3K, Akt, FKHR, and AFX. Moreover, cyclic stretch also causes the phosphorylation of stress-activated protein kinase/c-Jun N-terminal kinase. Attenuation in the level of phosphorylation of these proteins was observed by stretching cells in Ca^{2+} -free medium, using intra- (BAPTA-AM) and extracellular (BAPTA) calcium chelators, or gadolinium, suggesting that influx of extracellular calcium plays a significant role in the early response of osteoblasts to mechanical stimuli.

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Key words: Osteoblast; Stretch; Calcium; Akt; Stress-activated protein kinase/c-Jun N-terminal kinase

1. Introduction

The structure, organization, and tissue remodeling in bone is, at least in part, determined by its mechanical environment and loading history. However, the mechanism by which bone senses and processes mechanical input is poorly understood. Depending on the models used, mechanical strain results in increased osteoblast proliferation [1] or differentiation and mineralization [2–4]. In contrast, simulated microgravity results in osteoblast apoptosis [5].

The earliest response detected in mechanically stimulated bone cells is a rapid rise in intracellular calcium concentration ($[Ca^{2+}]_i$) [6]. Various types of mechanical stimuli including mechanical strain [7] elicit increases in $[Ca^{2+}]_i$ in osteoblasts. Consistent with these observations are patch clamp and imaging studies that have identified a stretch-activated (SA) chan-

nel in osteoblasts. Gadolinium (Gd^{3+}), which inhibits SA channels, significantly attenuates the increase in intracellular calcium concentration in mechanically stimulated osteoblasts [8,9]. Ca^{2+} conductance through these channels could trigger a number of second messenger responses that could ultimately be responsible for the anabolic effects of mechanical loading in bone. For example, calcium influx in osteogenic cells is essential for a mechanically induced increase in transforming growth factor β and prostaglandin E_2 [10], two molecules that play a major role in bone remodeling [11].

Recent studies have identified intracellular signaling pathways that mediate the biological effects associated with mechanical stimulation in vivo. In cardiac myocytes and other cell types, mechanical stimuli activate the phosphoinositide 3-kinase (PI3K) pathway, which results in protection from apoptosis through activation of the serine-threonine kinase Akt [12–15]. Akt (also called protein kinase B) regulates multiple biological functions, including gene expression, cell cycle transit, survival, cell transformation and oncogenesis (for a review see [16]). The mitogen-activated protein kinases (MAPKs) comprise a family of dual-specificity kinases that mediate the transduction of external stimuli into intracellular signals that regulate cell growth and differentiation. These include the extracellular signal-regulated kinase (ERK) pathway that is activated by mechanical stimuli in many cell types including osteoblast-like cells [17,18]. Another MAPK family member, c-Jun N-terminal kinase (JNK), is also activated by mechanical stretch in various cell types including periodontal ligament osteoblast-like cells [17,18]. In smooth muscle cells, calcium influx is essential for stretch-induced JNK activation [19].

Our study of mechanically stretched osteoblasts explores the early signaling events and their calcium dependence.

2. Materials and methods

2.1. Reagents

Gadolinium(III) chloride hexahydrate and bovine plasma fibronectin were both purchased from Sigma (St. Louis, MO, USA). The following monoclonal (mAb) and polyclonal (pAb) antibodies were used in this study: anti-phosphotyrosine 4G10 mAb (Upstate Biotechnology, Lake Placid, NY, USA), and anti-PI3K pAb (Upstate Biotechnology). The following antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA): anti-Akt pAb, anti-phospho-Thr₃₀₈-Akt pAb, anti-phospho-Ser₄₇₃-Akt pAb, anti-FKHR pAb, anti-phospho-Ser₂₅₆-FKHR pAb, anti-p44/42 MAPK pAb, anti-phospho-p44/42 MAPK (Thr₂₀₂/Tyr₂₀₄) pAb, anti-stress-activated protein kinase(SAPK)/JNK pAb, anti-phospho-SAPK/JNK (Thr₁₈₃/Tyr₁₈₅)

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pAb, anti-p38 MAPK pAb, anti-phospho-p38 MAPK (Thr₁₈₀/Tyr₁₈₂) pAb. The reporter antibodies used were: donkey anti-mouse or sheep anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA). Enhanced chemiluminescence reagents were purchased from Pierce. Ca²⁺-free α -modified Eagle's medium (α -MEM) was obtained from the Tissue Culture Core Facility, Harvard University, Boston, MA, USA.

2.2. Cell culture

MC3T3-E1 mouse clonal osteogenic cells [20] were maintained in α -MEM supplemented with L-glutamine, 10% fetal bovine serum (FBS; Intergen, Purchase, NY, USA), penicillin (100 IU/ml; Sigma), and streptomycin (100 mg/ml; Sigma).

2.3. Mechanical loading apparatus and cyclic stretch conditions

Cells were stretched as previously described [21,22] using the Cell Stretcher System NS 350H (Scholar Tech, Osaka, Japan). Twenty-four hours prior to stretching, cells were brought to a quiescent state by incubation in serum-free medium. The amplitude and the frequency of stretch were controlled by a programmable microcomputer at 20% stretch, 0.5 Hz for the times indicated. Cells were stretched in serum-free α -MEM supplemented, where indicated, with either BAPTA (1,2-bis(aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl)), the cell-permeable reagent BAPTA-AM or GdCl₃ (all reagents: Sigma) as follows: BAPTA at 5 μ M final concentration added at the time of stretching, BAPTA-AM at 5 μ M final concentration added 45 min prior to stretching and GdCl₃ at 10 μ M concentration added 15 min prior to stretching.

2.4. Measurement of cytosolic calcium

The concentration of [Ca²⁺]_i was measured as follows: osteoblasts on silicone membranes were incubated with the fluorescent calcium indicator FURA-2 [2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acid, methyl ester; Molecular Probes, Eugene, OR, USA] for 45 min and another 30 min in a solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM glucose, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), pH 7.40 as described previously [22]. [Ca²⁺]_i was measured by the FURA-2 method using a fluorescence microscope system (Arugas/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan) with a 20 \times objective (Zeiss, Fluor 20) as described previously [22]. Fluorescence ratio (*R*) was calculated from the following equation: $R = (F_{340} - B_{340}) / (F_{380} - B_{380})$, where *F*₃₄₀ and *F*₃₈₀ are the emission intensities at 510 nm excited at 340 and 380 nm, respectively, and *B*₃₄₀ and *B*₃₈₀ are corresponding autofluorescence values.

2.5. Immunoblotting

Cells were lysed in: 1% Triton X-100, 10 mM Tris pH 7.6, 500 mM NaCl, 2 mM sodium orthovanadate, 60 mM β -octylglucoside, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma). Proteins were then separated by SDS-PAGE and were transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated in blocking buffer [5% milk powder (Nestle Carnation, Frederick, MD, USA), phosphate-buffered saline (PBS)/Tween (0.5% Tween-20)] overnight at 4°C. All antibodies were diluted 1:1000 in blocking buffer. Immunoreactive proteins were visualized using the Pierce SuperSignal West Pico Chemiluminescent Substrate.

2.6. Immunoprecipitation

Aliquots of 50 μ l of Sepharose G bead slurry (Sigma) were incubated overnight with 50 μ g of anti-phospho-Tyr antibody or mouse IgG1 (Sigma). 200 μ g of cellular protein (obtained as described above) was added to 50 μ l of the antibody-treated Sepharose G bead slurry and incubated for an additional 2 h at 4°C. The lysates were then removed and beads were washed in lysis buffer and PBS. Proteins eluted by boiling in SDS sample buffer were then separated by SDS-PAGE and immunoblotted as described above.

3. Results

3.1. Effects of cyclic stretch on [Ca²⁺]_i in MC3T3-E1 cells

We confirmed that uniaxial stretch results in an increase in

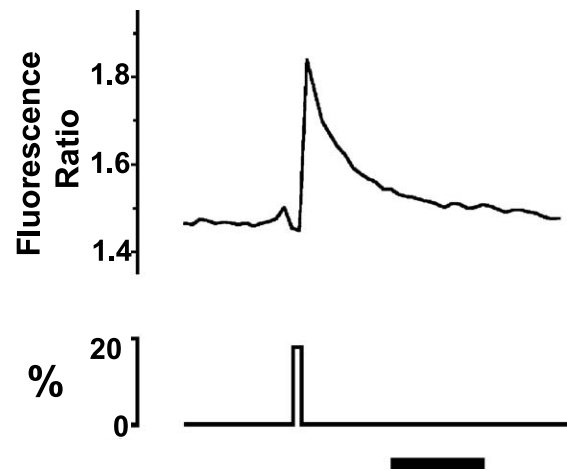


Fig. 1. Stretch-induced [Ca²⁺]_i increase in MC3T3-E1 osteoblasts. Osteoblasts in an elastic silicone chamber were subjected to a single stretch for 3 s and the change in [Ca²⁺]_i was measured as described in Section 2. The trace is a representative of five different experiments. The bar indicates 1 min.

[Ca²⁺]_i in MC3T3-E1 osteoblasts. Osteoblasts cultured on elastic silicone membrane were stretched for 3 s, and the change in [Ca²⁺]_i was measured immediately after stimulus application. As shown in Fig. 1, a single stretch could elicit a transient increase in [Ca²⁺]_i that declined to the initial basal [Ca²⁺]_i level.

3.2. Cyclic stretch induces phosphorylation of the anti-apoptotic PI3K/Akt pathway

Mechanical forces including shear stress [24] and stretch [13] result in Akt phosphorylation and activation in endothelial cells. Akt is an important downstream target of PI3K. We first investigated if PI3K becomes phosphorylated in response to stretch. Osteoblasts were stretched for 5, 10 and 30 min. Proteins immunoprecipitated with an anti-phosphotyrosine antibody were separated by SDS-PAGE and immunoblotted with an anti-PI3K antibody. Fig. 2A illustrates that cyclic stretch induces a time-dependent tyrosine phosphorylation of PI3K that is maximal at 5–10 min of stretch and returns to baseline by 30 min. We then used phospho-specific antibodies to investigate if Akt, the downstream target of PI3K, becomes phosphorylated in response to stretch. Fig. 2B demonstrates that cyclic stretch results in increased phosphorylation of Akt on Ser₄₇₃, a regulatory event that increases Akt kinase activity. A time-course study revealed that this phosphorylation is maximal at 10 min and decreases to baseline by 60 min (data not shown). Interestingly, Thr₃₀₈, the other major phosphorylation site involved in Akt activation, was not observed to be responsive to stretch (data not shown) consistent with published reports that regulation of phosphorylation at these two sites is not always equivalent [25–27]. Stretching osteoblasts in Ca²⁺-free medium decreased the observed Akt phosphorylation. When Ca²⁺ was sequestered by intracellular (BAPTA-AM) and extracellular (BAPTA) chelators, Akt phosphorylation was completely abrogated (Fig. 2B).

To test whether the stretch stimulus increased Akt activity in osteoblasts, we investigated phosphorylation of the transcription factors FKHR and AFX, two important substrates of Akt [28]. Fig. 2C (left panel) illustrates that a 10–30 min

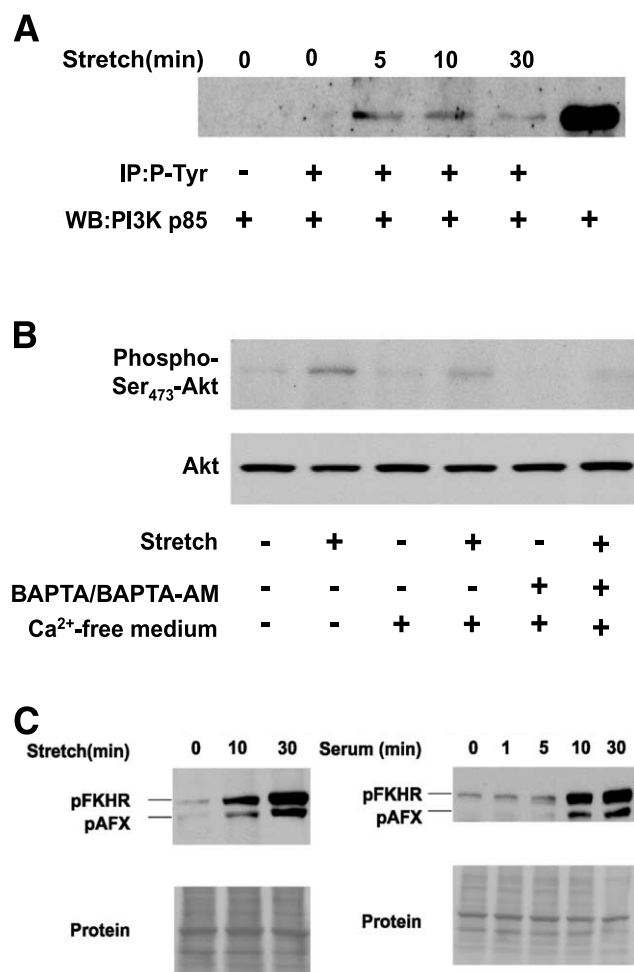


Fig. 2. A: Tyrosine phosphorylation of p85 PI3K in response to stretch. Osteoblasts were stretched for the times indicated. Proteins immunoprecipitated (IP) with an anti-phosphotyrosine-specific (IP: α P-Y) antibody were separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-PI3K antibody (WB). Lane 1: IP negative control (isotype-specific IgG); lane 6: immunoblotting positive control, MC3T3-E1 total cell lysate. B: Akt phosphorylation in response to stretch: the effect of BAPTA/BAPTA-AM and Ca²⁺-free medium. Osteoblasts were stretched for 10 min. Where indicated BAPTA and BAPTA-AM were added to Ca²⁺-free medium. 20 μ g of total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-phospho-Ser₄₇₃-Akt-specific antibody (upper panel) or anti-Akt antibody (lower panel). Calcium chelators abrogate completely the stretch-induced Akt phosphorylation while stretching in Ca²⁺-free medium decreases this phosphorylation. C: FKHR and AFX phosphorylation in response to stretch (left panel). Osteoblasts were stretched for 10 or 30 min. 40 μ g of total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-phospho-FKHR/AFX antibody (upper panel). The lower panel represents membrane staining with Ponceau S immediately after transfer and shows equal loading and transfer for all three lanes. FKHR and AFX phosphorylation in response to 10% FBS (right panel). Osteoblasts grown under similar conditions as for the stretch experiments were stimulated with 10% FBS for the times indicated. 20 μ g of total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-phospho-FKHR/AFX antibody (upper panel). The Ponceau S staining (lower panel) shows equal loading and transfer for all lanes.

stretch results in the phosphorylation of both FKHR and AFX, thereby confirming activation of a functional signaling cascade downstream of stretch-mediated Akt phosphorylation. Since serum is known to inhibit FKHR by phosphorylation [29], we stimulated osteoblasts grown in parallel with 10% FBS for up to 30 min. 10–30 min of serum stimulation results in the phosphorylation of both FKHR and AFX in a similar manner as stretch (Fig. 2C, right panel).

To extend our observation that [Ca²⁺]_i plays an important role in the stretch-induced phosphorylation of osteoblast proteins, we investigated pp60^{src}. One of us, K.N. [30], has previously shown that pp60^{src} becomes phosphorylated in response to mechanical strain in fibroblasts [30]. We demonstrated that osteoblast pp60^{src} becomes maximally phosphorylated in response to 10 min of stretch (data not shown) and that application of GdCl₃ or pretreatment with BAPTA/BAPTA-AM in a Ca²⁺-free medium, to chelate intracellular and extracellular calcium, inhibited the stretch-induced phosphorylation of pp60^{src} (data not shown).

3.3. Activation of MAPK family proteins in osteoblasts exposed to cyclic stretch

Further, we assessed activation of MAPK family proteins,

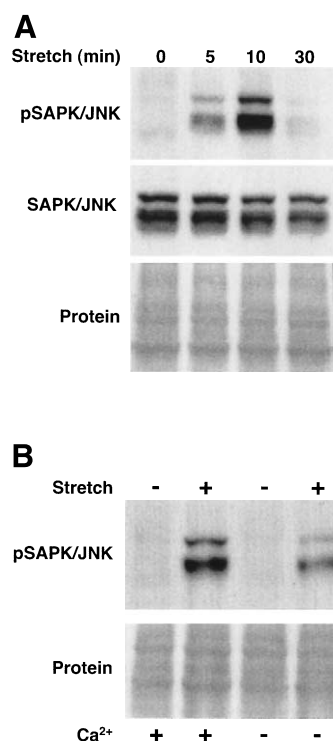


Fig. 3. A: SAPK/JNK phosphorylation in response to stretch. Osteoblasts were stretched for the times indicated. 10 μ g of total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-phospho-SAPK/JNK antibody (upper panel) or SAPK/JNK (middle panel). The lower panel represents membrane staining with Ponceau S immediately after transfer and shows equal loading and transfer for all lanes. B: SAPK/JNK phosphorylation in response to stretch is attenuated in Ca²⁺-free medium. Osteoblasts were stretched for 10 min in Ca²⁺-free medium. 10 μ g of total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-phospho-SAPK/JNK antibody (upper panel). The lower panel represents membrane staining with Ponceau S immediately after transfer and shows equal loading and transfer for all lanes.

which are also known to be stretch-responsive [31]. When osteoblasts were exposed to mechanical stretch for various times, phosphorylation of SAPK/JNK was increased to a maximal level by 10 min and returned to baseline by 30 min (Fig. 3A). This maximal phosphorylation was decreased when the cells were stretched in Ca^{2+} -free medium (Fig. 3B). We found no evidence of a stretch-induced phosphorylation of ERK or p38 for any of the times tested (data not shown).

4. Discussion

In the current study we have used an established mechanical stretch device [21,22] to show that in osteoblasts, uniaxial strain: (1) evokes an influx of extracellular Ca^{2+} , and (2) induces the phosphorylation of proteins that have been implicated in cell proliferation, survival and regulation of gene expression. Further, Ca^{2+} and stretch-activated channels are required for osteoblast protein phosphorylation in response to stretch. These findings provide the first demonstration of the dependence of the $\text{PI3K} \rightarrow \text{Akt} \rightarrow \text{FKHR}$, AFX pathway on $[\text{Ca}^{2+}]_i$ in stretched cells.

The mechanisms underlying mechanotransduction in bone cells are not understood in molecular terms. Altered Ca^{2+} flux has been implicated as a potential regulator of the response to mechanical signals in bone [32,33]. Following stretch application, we observed a rapid (within 30 s) rise in $[\text{Ca}^{2+}]_i$ levels consistent with previous studies in osteoblasts [6–8,23,34]. Ca^{2+} imaging and patch clamp studies in these cells have demonstrated Ca^{2+} flux through plasma membrane SA channels, which open in response to membrane deformation [6,9,23]. We found that the stretch-induced increase in Ca^{2+} could be inhibited in the presence of intracellular and extracellular chelators of ionic Ca^{2+} , and by gadolinium, an inhibitor of SA channels. These observations suggest that release of Ca^{2+} from internal stores and influx from the extracellular space both contribute to the rise in intracellular Ca^{2+} levels observed following mechanical stimulation of osteoblasts.

Since Ca^{2+} can trigger Akt phosphorylation [12], we investigated whether stretch results in osteoblast Akt activation and whether this activation is Ca^{2+} -dependent. In this study we show that mechanical stimulation of osteoblasts activates PI3K and its downstream target Akt, as well as two Akt-regulated transcription factors, FKHR and AFX [28]. Furthermore, under conditions of Ca^{2+} chelation, the stretch-induced phosphorylation of Akt was completely inhibited. These findings strongly implicate intracellular Ca^{2+} in regulating signaling through the PI3K/Akt pathway in osteoblasts subjected to mechanical strain. In their non-phosphorylated state, FKHR and AFX promote transcription of so-called death genes such as Fas ligand which mediate programmed cell death/apoptosis [29,35]. Akt-mediated phosphorylation of FKHR and AFX results in their export from the nucleus, thereby suppressing transcriptional activity and promoting cell survival [28]. Interestingly we observed that stretch and trophic factors (serum) elicited phosphorylation of FKHR/AFX in osteoblasts to similar extents, consistent with the hypothesis that stretch is a physiologically relevant regulator of bone physiology.

MAPK pathway activation by mechanical forces is highly cell type-dependent as various investigators have previously suggested [31]. In the current study we observed that osteoblasts exposed to strain display JNK/SAPK phosphorylation,

which is suppressed by inhibitors of Ca^{2+} flux. This observation is consistent with data generated in bladder smooth muscle cells [19]. Furthermore, we did not observe phosphorylation of other members of the MAPK family, namely ERK or p38. Our findings are consistent with the study by Matsuda et al. [17] in which stretch did not activate ERK or p38 in periodontal ligament osteoblast-like cells. JNK activation has been associated with growth arrest and apoptosis [36], however, we have no evidence to suggest that these occur. Since multiple pathways are responsible for a specific biological outcome, it is possible that uniaxial stretch activates various pathways such that the balance is in favor of osteoblast growth.

In summary, we have shown that stretch induces a rapid increase in $[\text{Ca}^{2+}]_i$ in osteoblasts which plays a key role in the phosphorylation and possible activation of various proteins, PI3K pathway members, and SAPK/JNK. These proteins play an essential role in regulating osteoblast survival, cell cycle transit, and differentiation. Future studies focusing on the characterization of Ca^{2+} -permeable stretch-activated channels in osteoblasts and the activation of downstream effectors of the proteins mentioned would improve our understanding of mechanotransduction in bone cells.

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