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β_1 Integrins Mediate Mechanosensitive Signaling Pathways in Osteocytes

Julie B. Litzenberger,

Department of Veterans Affairs, Bone and Joint Rehabilitation Center, Palo Alto, CA, USA;
Department of Bioengineering, Stanford University, Stanford, CA, USA

Padmaja Tummala,

Department of Veterans Affairs, Bone and Joint Rehabilitation Center, Palo Alto, CA, USA;
MedImmune Vaccines, Mountain View, CA, USA

Jae-Beom Kim, and

Department of Stomatology, University of California, Stanford, CA, USA; Caliper Life Sciences, Alameda, CA, USA

Christopher R. Jacobs

Biomedical Engineering Department, Columbia University, 500 W. 120th St., 351 Engineering Terrace, New York, NY 10027, USA

Abstract

Integrins are cell-substrate adhesion proteins that initiate intracellular signaling and may serve as mechanosensors in bone. MLO-Y4 cells were stably transfected with a dominant negative form of the β_1 integrin subunit (β_1 DN) containing the transmembrane domain and cytoplasmic tail of β_1 integrin. Cells expressing β_1 DN had reduced vinculin localization to focal contacts but no change in intracellular actin organization. When exposed to oscillatory fluid flow, β_1 DN cells exhibited a significant reduction in the upregulation of cyclooxygenase-2 gene expression and prostaglandin E_2 release. Similarly, the ratio of receptor activator of NF- κ B ligand mRNA to osteoprotegerin mRNA decreased significantly after exposure to fluid flow in control cells but not in β_1 DN cells. Interfering with integrin signaling did not affect mechanically induced intracellular calcium mobilization. These data suggest that integrins may initiate the cellular response of osteocytes to dynamic fluid flow and may serve as mechanosensitive molecules in bone.

Keywords

Integrin; Mechanotransduction; Osteocyte; Fluid flow; Bone

Bone is a dynamic tissue that adapts to its loading environment. Bone remodeling is a highly coordinated balance between bone-forming osteoblasts and bone-resorbing osteoclasts. These processes may be regulated by mechanosensitive osteocytes, terminally differentiated bone cells within the mineralized matrix [1]. The molecular mechanisms through which osteocytes sense mechanical stimuli are unknown. Integrins are extracellular matrix receptors that have been shown to be mechanosensitive in chondrocytes [2], cardiac

myocytes [3], and endothelial cells [4]. Identifying signaling pathways initiated by integrins in osteocytes is essential to understanding how bone tissue adapts to physical demands.

Integrins are heterodimeric transmembrane proteins, comprised of α - and β -subunits. Discrete contact points exist between osteocyte cell membranes and the mineralized bone matrix, and theoretical models predict that these connections are integrin-based [5,6]. Because integrin cytoplasmic domains are short and lack enzymatic function [7], activation of signaling cascades requires integrin binding to adaptor proteins and signaling molecules. Several *in vivo* studies support a role for integrins in bone mechanotransduction. Deletion of β_1 integrins from cortical osteocytes significantly reduces mechanically induced bone formation in a mouse ulna loading model [8], and mice with a bone-specific knockout of focal adhesion kinase (FAK), an integrin-mediated signaling molecule, lose their regenerative responsiveness to physical stimuli [9]. Young mice expressing function-perturbing β_1 integrin tails in mature osteoblasts have increased cortical porosity, decreased thickness, and lower bone formation [10], while adult transgenic mice have reduced bone mass, decreased femoral strength, and altered tibial curvature [11]. During hindlimb unloading, mice expressing a dominant negative β_1 integrin tail have a greater increase in cancellous resorption surfaces [12], and those with β_1 integrin deletion from cortical osteocytes experience larger changes in tibial cross-sectional geometry compared to controls [13]. *In vitro*, osteoblasts exposed to cyclic strain respond via Ras/Raf/MEK signaling, which is initiated by integrins [14]; and blocking focal adhesion assembly in cultured osteoblasts results in a decreased response to steady fluid flow [15] or compression [16]. While evidence exists that integrins mediate bone mechanosensitivity, the specific osteogenic pathways initiated by integrins in osteocytes exposed to dynamic loading have yet to be identified. The goal of this study was to identify integrin-mediated signaling pathways that are activated by mechanical loading.

In this study, MLO-Y4 osteocyte-like cells were induced to express a dominant negative fragment of the β_1 integrin (β_1 DN), containing the transmembrane domain and cytoplasmic tail of the β_1 integrin monomer. β_1 DN transgenic cells express both endogenous full-length β_1 integrins and β_1 DN fragments. Expression of β_1 cytoplasmic tails inhibits cell spreading, migration, matrix assembly, and FAK phosphorylation; and the β_1 DN fragment may competitively inhibit endogenous integrin function by binding to intracellular molecules that are crucial for a mechanosensitive response [17,18].

Several signaling pathways have been implicated in the tissue-level osteogenic response to mechanical loading. We examined markers of bone formation, bone resorption, and intracellular signaling, including cyclooxygenase-2 (COX-2), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG) mRNA; intracellular calcium ($[Ca^{2+}]_i$) concentration; and prostaglandin E₂ (PGE₂) production. COX-2 is involved in PGE₂ production [19], and blocking COX-2 or prostaglandins *in vivo* inhibits mechanically induced bone formation [20,21]. Although PGE₂ administration can increase both formation and resorption depending on infusion time course [22], loading-induced PGE₂ release appears anabolic through its action on the EP4 receptor [23]. Bone resorption requires the formation of mature, functional osteoclasts, which is regulated by relative levels of RANKL and OPG. RANKL stimulates osteoclast differentiation and activity [24], and its expression is affected by exposure to fluid flow [25]. OPG is a soluble factor that prevents RANKL from binding to its receptor, RANK [24], and bone resorption rate is believed to be affected by the relative ratio of these factors. MLO-Y4 cells exposed to dynamic fluid flow have been shown to modulate osteoclastogenesis, by direct cell-to-cell contact and release of soluble factors [26]. Finally, mechanically induced cell signaling was measured by quantifying $[Ca^{2+}]_i$. Ca^{2+} is a ubiquitous second messenger that regulates cellular metabolism. Dynamic fluid flow increases $[Ca^{2+}]_i$ in osteoblasts [27], which is required for

upregulation of osteopontin mRNA [28]. By measuring mechanically sensitive anabolic (COX-2, PGE₂), catabolic (RANKL, OPG), and intracellular signaling (Ca²⁺) pathways, we evaluated the cellular-level role of integrins in osteocyte mechanosensitivity.

This study aimed to implicate integrins as molecular transducers of physical stimuli in osteocytes and to identify particular osteogenic pathways initiated by integrins in bone. While in vivo experiments demonstrate the role of integrins in the tissue response to mechanical stimuli, it is critical to distinguish the intracellular pathways involved in this response. We hypothesized that β_1 DN cells would lose their ability to respond to loading-induced fluid flow through the COX-2, PGE₂, Ca²⁺, or RANKL and OPG pathways. We show that integrins mediate both anabolic and catabolic signaling in MLO-Y4 cells exposed to dynamic loading, further elucidating the role of integrins in osteocyte mechanosensitivity.

Materials and Methods

Expression of β_1 DN

MLO-Y4 parental cells were stably transfected with a CMV-driven expression vector encoding β_1 DN (Fig. 1a). β_1 DN cDNA contained the coding sequence for 70 amino acids of the endogenous cytoplasmic and transmembrane domains of the β_1 integrin and 20 amino acids of β_1 integrin signal peptide. The construct was marked by a Myc-epitope tag at the N terminus. β_1 DN cDNA was subcloned into *HpaI* and *HindIII* sites of the pLNCX expression vector (Clontech, Mountain View, CA). MLO-Y4 cells were transfected using the Effectene transfection kit (Qiagen, Valencia, CA) and screened using neomycin. Out of 29 clones screened, 19 clones were positive for β_1 DN expression, confirmed by Western blot analysis using the 9E10 myc-tag monoclonal antibody (Upstate Biotechnology, Charlottesville, VA). Myc-tag expression was monitored for 90 days by immunostaining to select the most stable clone. As a negative control, MLO-Y4 cells were stably transfected with an empty vector (vector control cells).

Cell Culture and Immunofluorescence

Cells were grown on tissue culture dishes coated with type I bovine collagen (BD Biosciences, Bedford, MA) in α MEM with 5% FBS and 5% CS (Hyclone, Logan, UT) and 1% Pen/Strep (Invitrogen, Carlsbad, CA). β_1 DN cells were immunostained for vinculin and actin to evaluate potential disruptions in focal adhesion assembly. Cells were incubated with mouse anti-vinculin primary antibody (Millipore, Bedford, MA; CBL233) overnight at 4°C, followed by 1 hour in AlexaFluor546 goat anti-mouse IgG (Invitrogen, A11030) with AlexaFluor488-conjugated phalloidin. Cells were imaged at 100 $\times$ on a TE-2000/C1 confocal microscope (Nikon Instruments, Melville, NY).

Oscillatory Fluid Flow

Forty-eight hours before experimentation, cells were sub-cultured on collagen-coated glass slides, which were then loaded into parallel-plate flow chambers for experiments. Cells were exposed to laminar oscillatory fluid flow (OFF) at 1 Hz and peak shear stresses of 1 Pa, as described previously [27,28]. Fluid flow rates were monitored using an ultrasonic flow meter (Transonic Systems, Ithaca, NY). No-flow (NF) controls were maintained by following the flow protocol without activating the flow-delivery system.

Western Blot

Total cellular lysate was obtained using RIPA buffer with protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA), and protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). For β_1 DN detection, we incubated membranes over-night at 4°C with a monoclonal anti-myc antibody, clone 9E10

(Upstate Biotechnology). To measure endogenous integrin expression, we used polyclonal anti- β_1 (1:200) primary mouse antibody (Santa Cruz Biotechnology), followed by 1 hour with HRP-conjugated anti-goat IgG (1:2,000 with Blotto). Signals were visualized using a chemiluminescent ECL substrate (GE Healthcare, Piscataway, NJ). Densitometric analysis was performed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Protein levels were normalized to β -actin in each sample.

Cell Adhesion Assay

A centrifuge-based cell adhesion assay was performed using an Eppendorf 5804 with a swing bucket rotor fitted with microplate buckets (A-2-MTP, effective radius 12.6 cm) [29]. Cells were subjected to detachment forces of either 140 $\times g$ or 1,268 $\times g$ for 10 minutes. Attached cells were counted with a hemocytometer immediately following centrifugation and normalized to the average total number of cells in each well before centrifugation.

Gene Expression

Cells were exposed to 2 hours of OFF at 37°C with 5% CO₂ and then lysed immediately with TRI® Reagent (Sigma-Aldrich, St. Louis, MO). A GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription. Real-time PCR was performed in the ABI Prism 7900HT Sequence Detection system using Taqman PCR Master Mix and 20X primers and probes for COX-2 (Mm00478374_m1), RANKL (Mm00441908_m1), OPG (Mm00435452_m1), and 18S (Hs99999901_s1) (Applied Biosystems). Each sample was measured in triplicate and normalized to 18S.

PGE₂ Release

Immediately after 2 hours of OFF, cell-seeded slides were removed from flow chambers and covered with 1 mL fresh medium. Cells were incubated at 37°C and 5% CO₂ for 1 hour. PGE₂ levels in the supernatant were measured using an enzyme immunoassay kit (GE Healthcare) and normalized to total protein, which was quantified with a BCA assay (Pierce, Rockford, IL).

Calcium Imaging

Real-time [Ca²⁺]_i was measured with 10 μ M Fura-2 AM fluorescent dye (Molecular Probes, Eugene, OR), using a ratiometric imaging protocol as described previously [27,28]. Images were collected every 2 seconds for a 3-minute baseline period, followed by 3 minutes of flow. We considered a cell as having responded to OFF if it experienced a transient increase in [Ca²⁺]_i of fourfold or more of the maximum concentration measured during the preflow baseline period. For each of the nine calcium experiments, we analyzed an average of 146 cells.

Statistical Analysis

One-way ANOVA was used to test for difference in means between multiple groups, followed by Student's *t*-test with a Bonferroni correction for multiple comparisons. For two-sample comparisons, Student's *t*-test was used. Statistical significance was accepted at *P* < 0.05, and data are reported as the mean $\pm$ standard error of the mean (SEM).

Results

β_1 DN Cells, but not Controls, Express β_1 Integrin Fragments

Expression of dominant negative β_1 integrin fragments in β_1 DN cells was confirmed by Western blot analysis (data not shown) and immunostaining using a myc-tag antibody (Fig. 1b). Vector control cells do not express β_1 DN, indicating that controls contain only

endogenous integrins, while β_1 DN cells contain both endogenous integrins and β_1 integrin fragments. There was no difference in endogenous β_1 integrin protein expression between β_1 DN and control cells ($P > 0.1$, $n = 4$), indicating that β_1 DN fragments do not affect endogenous β_1 integrin expression levels (Fig. 1c). Photomicrographs showed no morphological differences between β_1 DN cells and controls (data not shown). While the actin cytoskeleton of β_1 DN cells was not disrupted, vinculin localization to focal adhesions was diminished in β_1 DN cells (Fig. 1d).

Cell-Substrate Adhesion is Unaffected in β_1 DN Cells

Adhesion strength was not altered by transgene expression in β_1 DN cells. At $140\times g$, there was no significant difference in the number of cells attached to the substrate before versus after centrifugation for either cell type. At $1,268\times g$, however, 43% of β_1 DN cells and 51% of vector control cells detached from the substrate. There was no significant difference between β_1 DN cells and vector controls in the percentage of cells that remained adhered after centrifugation ($P > 0.1$ at both $140\times g$ and $1,268\times g$, $n = 6$ for each group) (data not shown). These data suggest that β_1 DN expression does not inhibit cellular binding to matrix proteins.

Inhibiting Integrin Signaling Reduces Mechanosensitivity of MLO-Y4 Cells to OFF

There was a sixfold increase in COX-2 gene expression in vector control cells in response to OFF ($P = 0.002$, $n = 4$) (Fig. 2a). In contrast, there was no significant change in COX-2 gene expression in β_1 DN cells in response to flow ($P > 0.1$, $n = 4$). Similarly, there was a significant increase in PGE₂ release in control cells ($P = 0.012$, $n = 7$) but not in β_1 DN cells ($P > 0.1$, $n = 7$) (Fig. 2b). The relative ratio of RANKL to OPG mRNA decreased significantly after exposure to OFF in vector controls ($P = 0.046$, $n = 6-8$) but not in β_1 DN cells ($P > 0.1$, $n = 6-8$) (Fig. 3). Absolute values of RANKL ($P > 0.2$, $n = 6-8$) and OPG ($P > 0.1$, $n = 6-8$) mRNA remained unchanged in transfected cells in response to OFF (data not shown). Interestingly, $(Ca^{2+})_i$ release was unaffected by β_1 DN expression in MLO-Y4 cells. An average 69% of vector control cells responded to OFF with increased $[Ca^{2+}]_i$ compared to 76% of β_1 DN cells (Fig. 4). The difference in percentage of cells responding between β_1 DN and control cells was not significant ($P > 0.1$, $n = 9$).

Discussion

Bone tissue alters its mass and structure to meet mechanical demands. Osteocytes may coordinate the cellular response to mechanical stimuli by releasing soluble factors and directing effector cells to deposit or resorb bone. Integrins serve as mechanosensors in many tissues, but their specific role in osteocyte mechanosensitivity is currently unknown. In this study, integrin signaling was activated by dynamic fluid flow, leading to an upregulation in markers of bone formation (COX-2, PGE₂) and a down-regulation in markers of bone resorption (RANKL, OPG).

In vitro data presented here are consistent with results from a β_1 DN mouse model in which β_1 DN mice exhibited less mineralized bone, reduced tibial curvature, and decreased femoral strength [10,11]. Additionally, β_1 DN mice had reduced cortical bone formation and increased cortical porosity, suggesting that β_1 integrins are important for normal bone growth [10]. In an ulnar loading model, mice with osteocyte-specific β_1 integrin deletion showed less cortical bone formation in response to 3 days of cyclic axial loading [8]. Our results extend these in vivo studies, establishing that integrins contribute to both anabolic (COX-2, PGE₂) and catabolic (RANKL, OPG) signaling in MLO-Y4 osteocytes exposed to dynamic loading. In this study, β_1 DN cells lost the ability to upregulate flow-induced COX-2 synthesis and PGE₂ release (Fig. 2). This pathway is crucial for the anabolic

response of bone to mechanical loading, and blocking COX-2 or prostaglandins in vivo inhibits mechanically induced bone formation [20,21]. These data suggest that integrins may influence bone mechanosensitivity in vivo via COX-2 and PGE₂ signaling. Interestingly, exogenous application of PGE₂ increases mRNA and cell surface expression of $\alpha_5\beta_1$ integrins in cultured primary osteoblasts [30], suggesting a potential for positive feedback.

Similarly, the ability of osteocytes to mediate osteoclastogenesis may be integrin-based. We show that β_1 DN cells have decreased inhibition of RANKL/OPG in response to OFF, which is consistent with prior findings that integrins are involved in the regulation of osteoclastogenesis. In cultured osteoblasts, RANKL expression is mediated by β_1 integrin-dependent adhesion to the bone matrix, and this regulates osteoclast maturation in coculture with peripheral monocytes [31]. Similar to our COX-2 and PGE₂ results, these data implicate integrins as molecular sensors that activate mechanically induced inhibition of osteoclastogenesis by osteocytes.

Interestingly, in response to hindlimb unloading, β_1 DN mice exhibited increased resorbing surfaces compared to controls [12], and mice with β_1 integrin deletion from cortical osteocytes showed augmented bone strength and stiffness, expansion of bone and marrow volumes, and increased inertial properties [13]. These data appear to contradict our finding that interfering with integrin signaling decreases loading-induced inhibition of osteoclastogenesis. A possible explanation for this discrepancy is that distinct mechanical stimuli may activate different integrin-mediated signaling pathways. Specifically, inhibiting integrin function may have an increased effect in response to unloading but a decreased osteogenic effect in response to loading.

β_1 DN expression did not alter actin organization or adhesion to collagen-coated substrate but did reduce vinculin colocalization to focal adhesions. Vinculin binding to integrins is critical for focal adhesion formation. Reduced vinculin colocalization to focal complexes suggests that β_1 DN fragments may competitively inhibit binding of signaling proteins to endogenous β_1 integrins. The actin network was unaffected in β_1 DN cells, indicating that β_1 DN expression did not compromise cytoskeletal integrity. Similarly, the adhesion of β_1 DN cells to collagen, which could affect cellular tension and the cellular response to fluid shear stress, was unaltered. These data suggest that β_1 DN expression inhibits intracellular signaling immediately downstream of integrins without significantly compromising the mechanical integrity of the cell.

We found that flow-induced $[\text{Ca}^{2+}]_i$ mobilization is unaffected by inhibited integrin signaling in MLO-Y4 osteocytes, which is consistent with previous results that blocking focal adhesion formation using GRGDS peptide does not affect the calcium response of an isolated primary osteocyte to steady fluid flow [32]. Surprisingly, in contrast to our findings, it has been reported that a low percentage of primary osteocytes respond to flow with increased $[\text{Ca}^{2+}]_i$ [32]. This discrepancy may be due to the demonstrated difference in the cellular response to dynamic versus steady fluid flow [27,33]. Also, the previous study analyzed the calcium response of isolated cells not in contact with adjacent cells. This does not reflect in vivo conditions, in which osteocytes can propagate intracellular calcium waves to adjacent cells [34].

Although COX-2 gene expression has been linked to $[\text{Ca}^{2+}]_i$ signaling in response to steady fluid flow in osteoblasts [35], COX-2 signaling occurs independently of $[\text{Ca}^{2+}]_i$ in response to dynamic fluid flow [36,37]. Our data are consistent with these previous findings. These results implicate integrins as the molecular switch in osteocytes that drives COX-2 and PGE₂ signaling in response to dynamic fluid flow, independent of $[\text{Ca}^{2+}]_i$ release.

In summary, β_1 DN expression in MLO-Y4 osteocytes blocked the upregulation of markers of bone formation (COX-2, PGE₂) in response to dynamic fluid flow. Similarly, integrin inhibition eliminated the flow-induced reduction in the ratio of RANKL to OPG, markers of bone resorption. While previous *in vivo* data have demonstrated a role for integrins in the tissue-level response of bone to mechanical stimuli, the data presented in this study are critical to identifying specific signaling pathways involved in this response. These data suggest that osteocytes may control the cellular response to mechanical stimuli by releasing both anabolic and catabolic factors and further implicate a role for integrins in the transmission of mechanical loads into the cell.

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Fig. 1.

Integrin signaling is inhibited in MLO-Y4 osteocyte-like cells. **a** MLO-Y4 cells are stably transfected with a blank vector (vector control cells, *left*) or a vector that induces expression of a dominant negative form of the β_1 integrin monomer (β_1 DN cells, *right*). **b** Stability of β_1 DN expression was confirmed by immunostaining for a myc-tag with the 9E10 antibody. Bar = 2 μ m. **c** Endogenous β_1 integrin expression is not affected by expression of the dominant negative integrin. Bars represent mean $\pm$ SEM ($n = 4$ for all groups). **d** Actin organization (*green—left*) is unaffected in β_1 DN cells (*bottom*), but vinculin (*red—middle*) colocalization to focal adhesions is diminished in β_1 DN cells compared to controls (*top*). Bar = 5 μ m. (color figure online)

Fig. 2. Inhibiting integrin signaling in MLO-Y4 osteocyte-like cells abrogates the upregulation of markers of bone formation in response to dynamic fluid flow. **a** COX-2 mRNA levels were quantified by real-time RT-PCR and normalized to 18S rRNA. **b** Extracellular PGE₂ levels were quantified by ELISA and normalized to total protein. Bars represent mean $\pm$ SEM ($n \geq 4$ for all groups, * significant difference between flow and no-flow groups $P < 0.05$)

Ratio of RANKL to OPG Gene Expression

Fig. 3.

Inhibiting integrin signaling in MLO-Y4 osteocyte-like cells blocks flow-induced reduction in markers of bone resorption. RANKL and OPG mRNA levels were individually quantified by real-time RT-PCR and normalized to 18S rRNA. The ratio of RANKL to OPG mRNA is plotted. Bars represent mean $\pm$ SEM ($n \geq 6$ for all groups, * significant difference between flow and no-flow groups $P < 0.05$)

Fig. 4. Intracellular calcium mobilization is unaffected by inhibited integrin signaling in MLO-Y4 osteocyte-like cells. The percentage of cells exhibiting Ca^{2+} flux during flow was measured by Fura 2-AM fluorescence ($n = 6$ for all groups). Bars represent mean $\pm$ SEM