Gardinier J, Yang W, Madden GR, Kronbergs A, Gangadharan V, Adams E, Czymmek K, Duncan RL. P2Y$_2$ receptors regulate osteoblast mechanosensitivity during fluid flow. Am J Physiol Cell Physiol 306: C1058–C1067, 2014. First published April 2, 2014; doi:10.1152/ajpcell.00254.2013.—Mechanical stimulation of osteoblasts activates many cellular mechanisms including the release of ATP. Binding of ATP to purinergic receptors is key to load-induced osteogenesis. Osteoblasts also respond to fluid shear stress (FSS) with increased actin stress fiber formation (ASFF) that we postulate is in response to activation of the P2Y$_2$ receptor (P2Y$_2$R). Furthermore, we predict that ASFF increases cell stiffness and reduces the sensitivity to further mechanical stimulation. We found that small interfering RNA (siRNA) suppression of P2Y2R attenuated ASFF in response to FSS or ATP treatment. In addition, RhoA GTPase was prevented by knocking down P2Y$_2$R. Finally, we confirmed that P2Y$_2$R activation of the RhoA GTPase signaling cascade, leading to asf and increased cell stiffness.

P2Y$_2$ receptor; osteoblast mechanosensitivity; cytoskeleton; Rho GT Pases; fluid flow

DYNAMIC LOADING of bone subjects osteoblasts to various forms of mechanical stimuli that induces osteogenesis and maintains bone homeostasis (5, 47). In vitro studies have been able to mimic these different types of mechanical stimuli that osteoblasts experience during dynamic loading of bone, such as substrate strain, hydraulic pressure, and interstitial fluid flow-induced shear stress (FSS) (17). Among the cellular changes that occur in response to FSS, reorganization of the actin cytoskeleton is thought to regulate cellular mechanosensitivity and mechanical behavior (4, 41). Moreover, changes in the actin cytoskeleton can also attenuate the anabolic response of osteoblasts during consecutive bouts of mechanical stimuli, although the cellular mechanism is unclear (14).

Restructuring of the actin cytoskeleton is largely dependent on purinergic signaling that has emerged as an essential component in osteoblast mechanotransduction (15, 24, 40). At the onset of FSS, osteoblasts release ATP and UTP stored in vesicles due to an influx of extracellular calcium through activation of mechanosensitive and voltage-sensitive calcium channels (11, 15, 45). This release of nucleotides activates purinergic receptors (P2), initiates several downstream markers of osteogenesis, such as c-Fos, ERK1/2, cyclooxygenase-2 (COX-2) expression, and PGE$_2$ release (3, 31). P2 receptors are classified as G protein-coupled receptors P2Y (P2Y$_{1,2,4,6,11,12,13,14}$) or nonselective, ligand-gated ion channels P2X (P2X$_{1-7}$) (39). The P2Y$_2$R and P2Y$_{13}$R knockout mice have both been shown to increase trabecular and cortical bone formation to dynamic loading, which suggests a negative regulation by the P2Y receptor in bone formation (40, 56). The negative feedback associated with the P2Y$_{13}$R is thought to be mediated by excessive ATP release (56). In contrast, the mechanism through which P2Y$_2$R downregulates the mechanosensitivity of bone is unknown, but we predict this receptor has a crucial function in regulating mechanotransduction.

Purinergic signaling has been associated with actin stress fiber formation (ASFF) and changes in cell stiffness, although the specific pathway that mediates ASFF in response to mechanical stimuli is poorly understood (14). FSS induces a rapid switch between actin filament polymerization and depolymerization that results in the reorganization of the actin cytoskeleton to form new stress fibers (1, 12, 32). The structural changes in the actin cytoskeleton contribute to the cell stiffness and can dictate the sensitivity of the cell to mechanical stimuli (7, 41, 57). As G protein-coupled receptors, P2Y receptors have the potential to modulate ASFF through their ability to activate the RhoA GTPase pathway (49). Upon RhoA GTPase activation, the downstream Rho kinase (ROCK) has been shown to phosphorylate LIM kinase-2 (LIMK-2) at Thr505 (1, 32, 49). Activated LIMK-2 mediates actin cytoskeleton reorganization in various cell types by phosphorylating, thereby inactivating, the actin-severing protein, coflin (16, 37, 52, 53). Coflin binding to actin promotes depolymerization of the actin filaments while phosphorylation of this protein enables actin polymerization and stress fiber formation (16, 37, 53). Overall, evidence suggests that the RhoA GTPase-activated ROCK/LIMK/cofilin pathway can regulate the actin cytoskeletal dynamics via a P2Y receptor in osteoblasts during mechanotransduction.

Several reports suggest that cells alter their apparent stiffness to enhance or protect themselves to mechanical stimuli (18, 25, 35). The loss of mechanosensitivity during continuous loading occurs at the cellular level in osteoblasts, as well as at the tissue level (9, 27). Adaptation in cell stiffness may also enhance the ability of cells to cope or even survive potentially injurious forces. Of the different cytoskeletal components, the F-actin and intermediate filaments appear to contribute the most to cell stiffness (20, 22). The ability of osteoblasts to alter their mechanosensitivity through actin cytoskeleton reorganization could have a large influence on the anabolic response (41, 57). While these data point to the cytoskeleton in defining the mechanosensitivity of the cell, the underlying mechanisms...
through which osteoblasts regulate their mechanosensitivity are not clearly understood. The purpose of our study was to identify the specific signaling mechanisms through which osteoblasts regulate their mechanosensitivity. The ASFF in response to FSS was hypothesized to be by P2Y2R-mediated activation of the RhoA GTPase and its downstream signaling pathway that, in turn, attenuates osteoblast mechanosensitivity.

**MATERIALS AND METHODS**

**Cell culture.** MC3T3-E1 osteoblast-like cells (Clone 14, ATCC, Bethesda, MD) were cultured in normal medium, which consisted of α-MEM, 10% FBS (GIBCO, New York, NY), 100 U/ml penicillin G, and 100 μg/ml streptomycin and buffered to pH 7.35 with 26 mM NaHCO3. Cells were maintained in a humidified incubator at 37°C and 100% humidity with 5% CO2-95% air and subcultured every 72 h. All cell culture media and antibiotics were purchased from Sigma Chemical, St. Louis, MO unless otherwise noted. For testing, cells were seeded at 1 × 104 cells/cm2 on glass slides coated with rat tail type I collagen (50 μg/ml in 0.02 N acetic acid, BD, Franklin Lakes, NJ), and experiments were conducted when the cells reached 80–90% confluency.

**FSS condition.** Cells were exposed to laminar fluid flow inside a parallel plate flow chamber using a gravity-driven closed flow loop that applied a shear stress of 12 dyn/cm² FSS as previously described (13). The apparatus was maintained at 37°C and the medium was aerated with 5% CO2-95% air during experiments. Cells were treated with 5 U/ml of apyrase (Sigma) in culture media before and during FSS to hydrolyze extracellular ATP and inhibit its downstream action. To determine whether RhoA GTPase mediated events during FSS, cells were pretreated with 10 μM ROCK inhibitor Y-27623 (35) and incubated for an additional 30 min at room temperature. After being washed with transfection media, the cells were incubated with the transfection mixture for 5 h at 37°C. Subsequently, the transfection mixture was replaced with normal media and the cells were allowed to grow.

**Suppression of P2Y2R siRNA.** To determine the role of P2Y2R in osteoblast mechanotransduction, we used a small interfering RNA (siRNA) strategy to suppress this receptor. Briefly, MC3T3-E1 cells were cultured in normal medium without antibiotics and grown to 60–70% confluency before transfection. A transfection mixture consisting of 100 μl of PEI (PolyPlus Transfection) or scrambled siRNA (C-siRNA) (Ambion) was added to each well, followed by HRP-labeled secondary antibody and the detection reagent. The optical density (OD) at 490 nm was measured to determine the magnitude of active RhoA GTPase in each sample. The degree of activation of RhoA GTPase was reported as a fold increase compared with static controls, which were not subjected to any form of treatment or mechanical stimuli. As a positive control for RhoA activation, cells were treated with 10 μM lysophosphatidic acid (LPA) for 30 min.

**RhoA GTPase activation.** The activation of RhoA GTPase in MC3T3-E1 cells was measured using a G-LISA kit (BK124, Cyto-skeleton). Following specified treatments, we collected cell lysates in the lysis buffer provided in the kit and immediately stored in liquid nitrogen for later use. To determine RhoA GTPase activation, 50-μl aliquots of each sample with a protein concentration of 1 mg/ml were added, in triplicate, to a 96-well plate. Anti-RhoA GTPase antibody was added to each well, followed by HRP-labeled secondary antibody and the detection reagent. The optical density (OD) at 490 nm was measured to determine the magnitude of active RhoA GTPase in each sample. The degree of activation of RhoA GTPase was reported as a fold increase compared with static controls, which were not subjected to any form of treatment or mechanical stimuli. As a positive control for RhoA activation, cells were treated with 10 μM lysophosphatidic acid (LPA) for 30 min.

**Ca2⁺ imaging with fluid flow.** MC3T3-E1 cells were grown for 3–4 days on type I collagen-coated (50 μg/ml in 0.02 N acetic acid) quartz slides to achieve 40–50% confluency. Before each experiment, cells were treated with siRNA to knock down P2Y2R expression. For flow experiments, cells were rinsed three times with Hanks’ balanced saline solution (HBSS) and then loaded with 3 μM Fura-2/AM (Molecular Probes, Eugene, OR), a fluorescent Ca2⁺ probe, in HBSS for 30 min at 37°C. Loaded cells were rinsed three times with HBSS and incubated for an additional 30 min with HBSS alone to ensure complete deesterification of the fluorescent molecule, yet minimize intracellular compartmentalization. A parallel-plate flow chamber with a uniform flow channel height of 250 μm was used to apply a fluid shear stress of 12 dyn/cm² to the cells, as previously described (21, 57). Laminar flow was introduced to the chamber through a syringe mounted on a Harvard Syringe Pump (PHD Programmable; Harvard Apparatus, Holliston, MA) that controlled the flow rate. To establish a fluid-flow intracellular Ca2⁺ concentration (125 mM) baseline, cells were exposed to fluid shear of 1 dyn/cm² for 3 min that was then increased to 12 dyn/cm² for 10–15 min. Flow was then stopped for several min to allow the cells to recover, after which the 12 dyn/cm² FSS was reintroduced. To determine the role of RhoA GTPase in this response, MC3T3-E1 cells were treated with 10 μM Y-27632 or 10 μM LPA while loading with Fura-2. Corresponding flow rates for each of the FSS levels were 1 and 15 ml/min, respectively. A ratemetric video-image analysis system (Immunocytometry Imaging, Cincinnati, OH) was used to record changes in [Ca2⁺]i. Fura-2 fluorescence was visualized with a Nikon inverted microscope using a Nikon ×10 fluoro objective. The cells were illuminated with a Xenon lamp equipped with quartz collector lenses. A shutter and filter changer containing the two different interference filters (340 and 380 nm) was computer controlled. In this system, emitted light was passed through a 430-nm dichroic mirror, filtered at 510 nm, and imaged with an integrating CCD video camera. The ratio of light emitted at 340-nm and 380-nm excitation was determined (F340/F380) from consecutive frames, and the [Ca2⁺]i for each cell was calculated from this ratio by comparison with fura-2 free acid standards. Computer-generated individual Ca2⁺ traces were population means derived from simultaneous recording of Ca2⁺ in the 10–15 single cells in the field of view. In response to both bouts of FSS, the [Ca2⁺]i of MC3T3-E1 cells treated with Y-27632 (10 μM for 60 min) or siRNA knockdown of P2Y2R were measured and compared with cells treated without drug treatment or the C-siRNA.

**Immunocytochemistry.** All immunofluorescence was carried out on cells fixed with 2% paraformaldehyde (Electron Microscopy Sciences) in PBS containing 0.1% Triton X-100 (Sigma) for 30 min on ice. Cells were then blocked with 3% BSA (Sigma) at room temperature for an hour and incubated with the respective primary antibodies. Rabbit polyclonal anti-phospho (Ser3) coflin (Abcam), rabbit polyclonal anti-phospho (Thr505) LIMK-2 (Abcam) was used to detect phosphorylation of coflin (P-coflin) and LIMK2 (P-LIMK-2), respectively. AF-488 phalloidin (Molecular Probes Biostatus) was used to label F-actin and DRAQ5 (Alexis) was used as a nuclear stain.
Slides were mounted using the Slow Fade Antifade kit and images were obtained using a Zeiss LSM510 laser scanning confocal microscope.

**Cellular stiffness.** The apparent stiffness of individual cells was measured using an Atomic Force Microscope (AFM) (BioScope II, Veeco) mounted on an inverted optical microscope. Soft microwell probes (MLCT-UANUM, Veeco) with a conical tip and a spring constant of 0.01 N/m were first calibrated using a thermal fluctuation method in fluid. Individual cells with normal morphology were then identified under the optical microscope and positioned under the probe. An area of 30 μm × 30 μm was first scanned at a speed of ~3 μm/s to generate a topographic map of an individual cell. Seven to ten points were selected over the cell body at the nuclear and perinuclear regions for indentation measurements. The peripheral region of the cell was avoided due to its relatively thin cell height and influence of the rigid substrate. At each selected point over the cell body, the cantilever tip indented the cell membrane at a speed of ~2.5 μm/s until a force of 100 pN was reached, which typically resulted in an indentation depth of <70 nm (10% of the cell height). The elastic modulus at each point was estimated from the recorded force-deflection curve using a Hertz-based model (42) as defined by the following:

\[ E = \frac{2F(1 - \nu^2)}{\pi\delta\tan(\phi)} \] (1)

where \( E \) is the apparent stiffness, \( F \) is the cantilever force measured by the AFM, \( \nu \) is the Poisson ratio of the cytoplasm (\( \nu = 0.4 \)) (33), \( \phi \) is the opening angle of the conical cantilever tip (\( \phi = 35^\circ \)). The indentation depth (\( \delta \)) was calculated by subtracting the cantilever deflection from the piezo displacement of the probe. The apparent stiffness for a given cell was defined by the average elastic modulus across each point measured. A total of five to six cells were measured following a given treatment, and each experiment was then repeated three times. The average cell stiffness for each treatment was compared with static controls and reported as a fold increase. Static controls were not subjected to any form of treatment or mechanical stimuli.

**Statistical analysis.** The change in cell stiffness and RhoA GTPase activation were reported as a fold increase (means ± SD) relative to static controls. Significance of all experiments was determined using one-way or two-way analysis of variance (ANOVA) with a Tukey post hoc test to determine significance when multiple comparisons in the study were made. Significance was defined by a \( P \) value < 0.05. Data for a given outcome measurement were pooled together in the case that no significant difference was found for a given treatment between different passages of cells.

**RESULTS**

**ASFF with application of FSS is due to P2Y2R.** We have previously demonstrated that in response to FSS, osteoblasts exhibit an increase in ASFF, which is abolished by hydrolysis of extracellular ATP with apyrase (14). In a dose-response study, we found that 100 μM of ATP or UTP contribute the most to ASFF compared with BzATP, which primarily activates P2X (Fig. 1A). The increased ASFF in response to FSS was also completely eliminated in the presence of apyrase (Fig. 1A). Based on these results, we hypothesized that the G protein-coupled receptor P2Y2 is responsible for ASFF in osteoblasts. To delineate its role in mechanotransduction, we suppressed P2Y2 in MC3T3 cells using siRNA. A transient suppression was successfully obtained, with significant knockdown occurring 48 h after transfection, followed by a full recovery to basal levels of protein synthesis 96 h after transfection as shown by Western blot (Fig. 1B). Hence, all the knockdown experiments were conducted 48 h posttransfection.

The downregulation of P2Y2 receptors greatly reduced the formation of actin stress fibers to FSS applied for 60 min. Similar results were observed when these cells were treated with 100 μM ATP treatment for 30 min (Fig. 1C), indicating that the activation of the P2Y2 receptor is required for FSS-induced stress fiber formation. To ensure the transfection reagents used in the process did not alter the normal function of the cell, a C-siRNA was used. No significant difference was found on ASFF in response to FSS or ATP in cells treated with C-siRNA compared with nontransfected cells.

**P2Y2R is required for the activation of RhoA GTPase during FSS to form stress fibers.** Shear stress initiates numerous cellular events in osteoblasts that eventually lead to protein expression required for bone formation. Among the early cellular events is the rapid reorganization of the actin cytoskeleton and formation of stress fibers. We postulate that this active reorganization will increase cell stiffness, making MC3T3-E1 cells less pliable, and therefore less responsive, to continued mechanical stimulation. The formation of actin stress fibers was one of the initial functions attributed to the small GTPase RhoA but has also been found to be involved in a variety of signaling processes. Using the G-LISA kit, we found that FSS increased the active form or GTP-bound RhoA in MC3T3-E1 cells, as measured by an increase in absorbance at 490 nm. The graph in Fig. 2A shows that RhoA GTPase is activated within 5 min of the onset of FSS with peak activation (~4-fold increase) observed at 15 min. This increase was similar to the approximately sixfold increase achieved by treatment with 10 μM LPA, a potent activator of RhoA GTPase.

We further demonstrated that the purinergic receptor P2Y2 is required for FSS-induced RhoA GTPase activation in MC3T3-E1 cells (Fig. 2B). The fold change in active RhoA GTPase after 60 min of FSS or 30 min of 100 μM ATP stimulation in P2Y2R knockdown cells was significantly attenuated compared with nontransfected or C-siRNA cells. To determine whether RhoA GTPase activation via the P2Y2R is essential for the formation of stress fibers, the downstream effector of RhoA GTPase ROCK was blocked with Y-27632 before FSS. AF-488 phallolidin staining of actin stress fibers showed that FSS-induced ASFF was completely prevented by inhibiting ROCK, whereas activation of RhoA GTPase by LPA caused a pronounced increase in ASFF (Fig. 2C).

**RhoA GTPase activation during FSS initiates the ROCK/LIMK/cofilin pathway.** Numerous studies have shown that the ROCK/LIMK/Cofilin pathway is strongly affiliated with stress fiber formation in many cell types. A serine-threonine protein kinase LIM-K is activated by several proteins including ROCK, and cofilin is one of the primary substrates of this enzyme. Upon phosphorylation, cofilin is rendered inactive and hence unable to bind and perform its function of severing actin filaments, thereby stabilizing actin stress fibers. Immunofluorescence data indicates that LIMK-2 is phosphorylated at Thr505 within 15 min of the onset of FSS, and this phosphorylation directly correlates with an increase in P-cofilin (Fig. 3). Similar results were achieved when RhoA GTPase was activated using LPA. However, only faint staining of P-cofilin was observed under static conditions or when the ROCK inhibitor Y-27632 was used before FSS. These photomicrographs indicate that the ASFF induced by FSS in osteoblasts is mediated...
by the activation of the ROCK/LIMK/Cofilin pathway by RhoA GTPase as illustrated in Fig. 6.

P2Y2R regulates changes in cell stiffness to mechanical stimuli in osteoblasts. The mechanosensitivity of a cell is largely dependent on the structural integrity of its actin cytoskeleton. Increase in actin stress fibers is known to elevate the overall cell stiffness and thereby thought to potentially decrease the mechanosensitivity (19, 20). Here we demonstrate that the purinergic receptor P2Y2 regulates ASFF and, thereby, osteoblast cell stiffness by activating RhoA GTPase. Osteoblasts subjected to 15 min of FSS exhibited an approximately sixfold increase in cell stiffness as measured by AFM. To determine whether RhoA GTPase is involved in this increase in stiffness, cells were first treated with 10 μM LPA for 15 min to activate RhoA GTPase. LPA treatment produced an approximately fivefold increase in cell stiffness (Fig. 4A).

Inhibition of ROCK during FSS with 10 μM Y27632 significantly blocked any changes in cell stiffness, implicating RhoA GTPase in the regulation of cell stiffness during FSS.

Given the role P2Y2R has in mediating ASFF through RhoA, we suppressed P2Y2R expression with siRNA that significantly reduced the increase in cell stiffness following 15 min of FSS or 30 min of treatment with ATP (Fig. 4B). Overall, these results provide a cogent argument that P2Y2R-mediated activation of RhoA GTPase is critical for the increase in cellular stiffness and potentially, regulation of mechanosenstivity.

Increase in cellular stiffness due to ASFF during FSS reduces the mechanosensitivity of osteoblasts to consecutive stimulation. Osteoblast adaptation to mechanical stimulation was determined based on the [Ca²⁺]i response to successive bouts of FSS. The peak [Ca²⁺]i response of untreated cells to the initial bout of FSS was approximately three times greater than basal [Ca²⁺]i levels, with over 80% of the cells responding with a 0.5-fold or greater increase in [Ca²⁺]i (Fig. 5, C and D). The [Ca²⁺]i response to a second bout of FSS was significantly smaller in untreated cells, with peak [Ca²⁺]i just ~1.5 times greater than basal [Ca²⁺]i levels. Furthermore, less than 5% of cells responded to this second bout of shear (Fig. 5, C and D). These data are in accordance with the physiology of the bone and the law of diminishing returns to mechanical load and provides evidence for the desensitization of osteoblasts to physical stimuli (5). We found that inhibition of ROCK with
Fig. 2. RhoA GTPase is activated during FSS to form stress fibers due to the activation of P2Y2R. A: RhoA GTPase was measured in MC3T3 cells at different time points following the onset of FSS (12 dyn/cm²). Treatment with 1 μM lysophosphatidic acid (LPA) under static conditions elicited a maximal activation of RhoA GTPase (~6-fold). The maximum increase in RhoA GTPase activation during FSS occurred 15 min after the onset of stimuli and was approximately four times greater than compared with static controls (a indicates P value < 0.05 compared with static control). B: G-LISA assay was used to measure RhoA GTPase activation in response to ATP treatment and FSS in nontransfected cells and cells transfected with P2Y2R siRNA. The peak RhoA GTPase activation occurred 15 min after the onset of either ATP treatment or FSS in nontransfected cells. This peak was nearly abolished with the suppression of P2Y2R. Cells transfected with C-siRNA did not exhibit any significant differences compared with nontransfected cells. *P value < 0.05 compared with static control. C: MC3T3-E1 cells, stained for F-actin, exhibited a diffuse organization of actin under static conditions compared with cells exposed to FSS (12 dyn/cm²) for 60 min. LPA treatment (10 μM for 30 min), which activates RhoA GTPase, significantly increased the ASFF. Inhibition of ROCK with 10 μM Y27632 during FSS significantly inhibits ASFF. Bar indicates 20 μm.
Y27632 and siRNA knockdown of P2Y2R significantly increased the peak $[\text{Ca}^{2+}]_i$ during the first response to FSS, although it did not affect the number of cells responding (Fig. 5, C and D). Furthermore, both ROCK inhibition and siRNA knockdown of P2Y2R also significantly increased the peak $[\text{Ca}^{2+}]_i$ and number of responding cells compared with untreated controls during the second bout of FSS (Fig. 5, C and D). Based on these results, ROCK mediates ASFF via the P2Y2 receptor activation during FSS. Hence, the activation of ROCK through RhoA GTPase is able to attenuate the $[\text{Ca}^{2+}]_i$ response in osteoblasts to successive bouts of FSS, providing strong evidence for its function in regulating osteoblast mechanosenstivity. Although ASFF mediated by RhoA GTPase does not become visually evident until 30 min after the onset of FSS, the loss of mechanosensitivity was apparent at 15 min, as observed by the lack of $[\text{Ca}^{2+}]_i$, response to subsequent bouts of FSS.

Fig. 3. Activation of the Rho kinase (ROCK)/Lim kinase (LIMK)/cofilin pathway during FSS to form ASFF in osteoblasts. The presence of phosphorylated cofilin (P-cofilin) (right) was increased after 15 min treatment with 1 μM LPA under static conditions, as well as after 15 min of FSS (12 dyn/cm²). Inhibition of ROCK during FSS with 10 μM Y27632 attenuated the presence of P-cofilin. A similar response was observed in LIMK-2 phosphorylation under the same conditions (left). Bar indicates 20 μm.
The rapid loss of sensitivity to shear can be, at least in part, attributed to the initial formation of stress fibers and increase in cell stiffness.

**DISCUSSION**

We have demonstrated that activation of the P2Y2R receptor is required for the actin cytoskeletal rearrangement in response to FSS in vitro. Our findings show that RhoA GTPase was activated in response to FSS and is downstream of P2Y2R activation. Our data indicate that changes in cell stiffness to physical stimuli in osteoblasts are dependent on the P2Y2R and RhoA GTPase signaling pathway. In this study, we suggest a novel mechanism by which purinergic signaling can regulate osteoblast mechanosensitivity in vitro.

The mechanical forces generated in bone during dynamic loading stimulates purinergic signaling in osteoblasts and is crucial to osteogenesis (39). The vesicular release of ATP is highly regulated by the influx of intracellular calcium through mechanosensitive and voltage-sensitive channels (11, 15, 45, 46). Extracellular ATP released during mechanical stimulation then activates purinergic receptors (P2) in an autocrine/paracrine fashion. Purinergic signaling then results in the immediate activation of calcium signaling. In this study, we investigated the role of purinergic signaling in osteoblast mechanosensitivity.

**Material and Methods**

We examined the role of purinergic signaling in osteoblast mechanosensitivity using immortalized osteoblastic MC3T3-E1 cells. The effects of purinergic signaling on osteoblast mechanosensitivity were assessed by measuring cell stiffness in response to FSS. Cell stiffness was measured using atomic force microscopy (AFM) under static conditions and in response to FSS. The effects of purinergic signaling on cell stiffness were assessed by measuring the peak force applied to the cells during FSS.

**Results**

The rapid loss of sensitivity to shear can be, at least in part, attributed to the initial formation of stress fibers and increase in cell stiffness.

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P2Y2 REGULATES OSTEOBLAST MECHANOSENSITIVITY

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We have previously postulated that altering cell stiffness may be one mechanism through which parathyroid hormone treatment can enhance mechanically stimulated bone formation (48, 57).

Our data indicate that the P2Y2R facilitates changes in the mechanical behavior of osteoblasts through increasing ASFF and cell stiffness. The P2Y2R is a G protein-coupled receptor that can be activated by ATP or UTP (40). In this study, we show that the P2Y2R activates RhoA GTPase, probably through G protein activation of a Rho guanine exchange factor. We also show that activation of RhoA GTPase regulates the response of osteoblasts to FSS, similar to other cell types that experience various forms of mechanical stimuli (6, 12, 55). In response to FSS, RhoA GTPase activation in osteoblasts was evident as early as 5 min after the onset of stimuli and reached peak activation at 15 min. The activation of RhoA GTPase within 5 min of the onset of FSS coincides with the extracellular concentration of ATP being at its highest level based on previous studies (15).

We show that actin is rapidly reorganized into stress fibers in response to FSS and that this occurs through stimulation of the RhoA GTPase pathway. Other cell types have also been shown to regulate cytoskeleton organization through activation of RhoA GTPase (12, 32, 52, 53). Stress fiber formation is permitted once cofilin is phosphorylated and dissociates from actin filaments to allow actin polymerization. Phosphorylation of cofilin can occur through activation of the LIM kinase family, which consists of LIMK-1 and LIMK-2, by phosphorylation at Thr508 or Thy505, respectively (26, 30, 53). LIMK-1 and LIMK-2 are closely related serine-threonine kinases, whose structures contain two NH2-terminal LIM domains, an internal PDZ domain, and a COOH-terminal protein kinase domain (1, 16, 50). However, LIMK-2 is found more readily in embryonic and adult tissue compared with LIMK-1 (51). Similar to previous studies, our results demonstrated that LIMK-2 is activated downstream of RhoA GTPase and phosphorylated at Thr505 (1, 52). Phosphorylation of LIMK-2 has been shown to be an essential component of actin cytoskeleton dynamics in many cell types, and in this study we have shown...
it to be associated with ASFF during mechanical stimuli of osteoblasts.

The rapid activation of RhoA GTPase following 5 min of FSS was sufficient to downregulate the sensitivity of osteoblasts to a second bout of FSS 10 min after the first stimulation. Although ASFF is not visually evident until 30 min after the onset of FSS, the mechanosensitivity was reduced much earlier, based on the lack of a [Ca\(^{2+}\)]\(_i\) response to a second bout of FSS. One possibility for this temporal discrepancy is that RhoA GTP is geranylgeranylated to the plasma membrane; thus cortical actin found beneath the plasma membrane would be a probable early target of RhoA GTPase activation. This cortical actin regulates membrane stiffness and anchors many membrane proteins, including ion channels that would alter Ca\(^{2+}\) signaling (43). Another study has reported that an initial bout of FSS, lasting only 2 min, did not affect the subsequent [Ca\(^{2+}\)]\(_i\) response to a second bout of FSS (9). The ability of osteoblasts to retain their mechanosensitivity following only 2 min of FSS may be due to a lack of time for the cell to respond with activation of RhoA GTPase. Given the results from our study, osteoblasts appear to exhibit a threshold for prolonged mechanical stimuli that is between 2 and 5 min, after which they begin to downregulate their mechanosensitivity.

The temporal aspects of mechanical loading are known to have a large influence on the anabolic response of bone at the tissue and cellular levels (5, 51). Despite several advances in understanding mechanotransduction in osteoblasts, little is known about the cellular mechanism involved in regulating mechanosensitivity of osteoblasts. In this study, we provide new insights into a possible regulatory mechanism to physical stimuli. Figure 6 illustrates the cascade of events when FSS is applied to osteoblasts. The physical stimulus triggers the opening of the mechanosensitive cation channel (MSCC), which subsequently opens L-type voltage-gated calcium channels (L-VSCC) (8, 10, 28, 48). This increase in [Ca\(^{2+}\)]\(_i\) induces the vesicular release of ATP into the extracellular space, which then activates purinergic receptors, specifically the P2Y\(_2\)-R (15, 31, 46). Based on our results, the P2Y\(_2\)-R then activates RhoA GTPase and consequently activates ROCK, which culminates in the actin reorganization to form stress fibers via the LIM-K/cofilin pathway. In parallel with the RhoA/ROCK/LIM-K/cofilin pathway, P2Y\(_2\)-R activation has also been shown to initiate intercellular calcium release from the ER that is linked with ASFF as well (8, 23). Increased ASFF stiffens the cells, consequently attenuating the ability of osteoblasts to detect further mechanical stimulus and arresting the downstream signaling that would lead to osteogenesis. Robling et al. (44) have reported that mechanically induced osteogenesis can be enhanced with a break in between loading bouts, suggesting that the ability of the cells responding to physical stimuli is only temporarily attenuated. We propose that formation of ASFF via P2Y\(_2\)-R activation is a way to desensitize osteoblasts, which can potentially regain its sensitivity over time. Based on our findings, we predict that the increased bone formation observed in the P2Y\(_2\)-R knockout mice can be partly attributed to the continuous signaling to the physical stimuli (40).

In summary, our findings suggest a novel mechanism by which the purinergic receptor P2Y\(_2\)-R can regulate osteoblast mechanosensitivity. Furthermore, we directly implicate P2Y\(_2\)-R to ASFF in response to FSS through a RhoA GTPase-dependent mechanism that, in turn, activates the ROCK/LIMK/cofilin pathway. Additionally, our data indicate that the increase in cell stiffness in response to FSS is also mediated by the P2Y\(_2\)-R receptors. Finally, our findings suggest that by blocking ROCK and the eventual ASFF during FSS, the loss in mechanosensitivity of the osteoblasts can be averted.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


