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Rac1 and Cdc42 GTPases regulate shear stress-driven β-catenin signaling in osteoblasts

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ABSTRACT

Beta-catenin-dependent TCF/LEF (T-cell factor/lymphocyte enhancing factor) is known to be mechanosensitive and an important regulator for promoting bone formation. However, the functional connection between TCF/LEF activity and Rho family GTPases is not well understood in osteoblasts. Herein we investigated the molecular mechanisms underlying oscillatory shear stress-induced TCF/LEF activity in MC3T3-E1 osteoblast cells using live cell imaging. We employed fluorescence resonance energy transfer (FRET)-based and green fluorescent protein (GFP)-based biosensors, which allowed us to monitor signal transduction in living cells in real time. Oscillatory (1 Hz) shear stress (10 dynes/cm^2) increased TCF/LEF activity and stimulated translocation of β-catenin to the nucleus with the distinct activity patterns of Rac1 and Cdc42. The shear stress-induced TCF/LEF activity was blocked by the inhibition of Rac1 and Cdc42 with their dominant negative mutants or selective drugs, but not by a dominant negative mutant of RhoA. In contrast, constitutively active Rac1 and Cdc42 mutants caused a significant enhancement of TCF/LEF activity. Moreover, activation of Rac1 and Cdc42 increased the basal level of TCF/LEF activity, while their inhibition decreased the basal level. Interestingly, disruption of cytoskeletal structures or inhibition of myosin activity did not significantly affect shear stress-induced TCF/LEF activity. Although Rac1 is reported to be involved in β-catenin in cancer cells, the involvement of Cdc42 in β-catenin signaling in osteoblasts has not been identified. Our findings in this study demonstrate that both Rac1 and Cdc42 GTPases are critical regulators in shear stress-driven β-catenin signaling in osteoblasts.

1. Introduction

Mechanical loading of bone is known to influence bone growth and remodeling [1,2]. Application of physiological loading to bone accelerates bone formation and fracture healing, whereas removal of loading results in bone loss [3,4]. The process of growth and remodeling of bone involves the coordinated activity of two types of cells present in bone: bone-forming osteoblasts and bone-resorbing osteoclasts. In osteoblasts, β-catenin-dependent Wnt signaling is known to be one of the important regulators to promote bone formation [5] and its activation is regulated by mechanical loading including fluid flow-induced shear stress [6,7] and biaxial stretching [8]. In response to mechanical loading, β-catenin in the cytoplasm is stabilized by the inactivation of a destruction complex such as axin and GSK3β (glycogen synthase kinase 3β) and translocated to the nucleus. The β-catenin in the nucleus associates with TCF/LEF (T-cell factor/lymphocyte enhancing factor) transcription factors, leading to the activation of TCF/LEF and induction of expression of Wnt target genes [9]. Although the regulatory role of mechanical loading in β-catenin signaling activity is well defined, the precise mechanism by which how β-catenin signaling interacts with other mechanoresponsive signaling proteins, such as Rho family GTPases, is not clearly understood.

The Rho family GTPases act as a molecular switch that regulates fundamental processes including morphogenesis, polarity, movement, and cell division [10]. They present an active (GTP-bound) and inactive (GDP-bound) states, which are controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). RhoA, Rac1, and Cdc42 are most well studied members of the GTP-binding proteins and are known to play distinctive roles in regulating actin cytoskeletal dynamics of stress fibers, lamellipodia, and filopodia, respectively [11]. Accumulating evidence indicates that they respond to mechanical loading in various cell types, including osteoblasts [12], smooth muscle cells [13], endothelial cells [14], and mesenchymal progenitor cells [15]. Moreover, the Rho family GTPases are involved in bone resorption by regulating osteoclast functions [16–18].

Here we report, for the first time, that Rac1 and Cdc42 are critical components of β-catenin signaling in shear stress-induced

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osteoblasts. Since the activities of these GTPases can be rapid and transient [13,19], we employed fluorescence resonance energy transfer (FRET) technique and live cell imaging approach to capture their dynamic patterns. We applied oscillatory fluid flow-induced shear stress to MC3T3-E1 cells and imaged the activities of the individual GTPases and TCF/LEF as well as β-catenin translocation at high spatiotemporal resolution. To examine the role of the individual GTPases in mediating shear stress-induced TCF/LEF activity, we used constitutively active and dominant negative GTPase mutants as well as GTPase-specific pharmacological drugs. The roles for cytoskeletal structures and myosin activity in shear-stress-induced TCF/LEF activity were also examined.

2. Materials and methods

2.1. Rho family GTPase biosensors and mutants

FRET-based GTPase biosensors for monitoring Rac1, Cdc42, and RhoA were used [20,21]. Each biosensor contains two fluorescent proteins, cyan fluorescent protein (CFP) as the FRET donor and yellow fluorescent protein (YFP) as the FRET acceptor. One of the GTPases (Rac1, Cdc42, or RhoA) and a binding domain of an effector protein for the specific GTPases (p21 protein-activated kinase 1 for Rac1 or Cdc42, and protein kinase N for RhoA) are inserted between the CFP and YFP. An activated GTPase promotes the intramolecular binding of the GTPase to a specific binding within the biosensor, which results in the close association of CFP with YFP, and an increase of FRET from CFP to YFP. The YFP/CFP emission ratio was used as a measure of the specific GTPase activity. The biosensors have been well characterized in terms of its specificity [12,13,20,21]. As GTPase mutants, constitutively active Rac1 (Rac1-L61) and Cdc42 (Cdc42-L61), as well as dominant negative Rac1 (Rac1-N17), Cdc42 (Cdc42-N17), and RhoA (RhoA-N19) were used [22,23].

2.2. TCF/LEF reporter and β-catenin probe

A TCF/LEF-green fluorescent protein (GFP) reporter (SA Biosciences, Valencia, CA, USA) was used to monitor the activity of β-catenin signaling since β-catenin activation is known to lead to TCF/LEF transcriptional activation [24]. The TCF/LEF reporter utilizes the inducible transcription factor-responsive GFP and increases the GFP intensity level in the cytoplasm when activated β-catenin forms a complex with TCF/LEF transcription factors. Enhanced GFP (EGFP)-β-catenin fusion proteins were used to monitor translocation of β-catenin [25].

2.3. Cell culture and transfection

MC3T3-El osteoblasts were used for this study (ATCC, Manassas, VA, USA). The cells were cultured in minimum essential alpha medium (αMEM; Invitrogen, Grand Island, NY, USA) containing 10% FBS (Invitrogen) and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin; Lonza, Basel, Switzerland). Prior to experiments, the cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. The DNA plasmids were transfected into the cells using a Neon transfection system (Invitrogen). After transfection, the cells were transferred to a type I collagen-coated μ-slide cell culture chamber (Ibidi, Martinsried, Germany) and incubated in αMEM containing 0.5% FBS for 24–36 h before imaging experiments.

2.4. Pharmacological drugs

NSC 23766 (50 μM; Tocris Bioscience, Bristol, UK) was used to prevent Rac1 activation. ML141 (10 μM; Tocris Bioscience) was used to inhibit Cdc42 activity. Rac/Cdc42 activator II (100 ng/ml) was from Cytoskeleton (Denver, CO, USA). XAV939 (1 mM; R&D Systems, Minneapolis, MN, USA) was used to inhibit β-catenin signaling. Cytochalasin D (1 μg/ml; Enzo Life Sciences, Farmingdale, NY, USA) was used to disrupt actin filaments. Nocodazole (1 μM; Sigma–Aldrich, St. Louis, MO, USA) was used to disrupt microtubules. Blebbistatin (50 μM; Toronto Research Chemicals, Toronto, ON, Canada) was used to specifically inhibit myosin II activity.

2.5. Shear stress application

To apply oscillatory (1 Hz) flow to the MC3T3-E1 cells, an OsciFlow controller (Flexcell International, Hillsborough, NC, USA) was used. Shear stress of 10 dynes/cm^2 was applied to the cells grown in the μ-slide cell culture chamber (Ibidi) by controlling the flow rate of a peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA). Two pulse dampeners (Cole-Parmer) were connected to both the inlet and outlet of the cell culture chamber to minimize pulsation of the flow. During flow experiments, the flow system was perfused with HEPES-buffered (20 mM) αMEM without serum to maintain the pH at 7.4. The flow experiments were conducted at 37 °C.

2.6. Microscopy and image analysis

A Nikon Ti-E inverted microscope equipped with a charge-coupled device (CCD) camera (Evolve 512; Photometrics, Tucson, AZ, USA) and a filter wheel controller (Sutter Instruments, Novato, CA, USA) was used for imaging experiments. The following filter sets (Semrock, Rochester, NY, USA) were used: CFP excitation: 438/24 (center wavelength/bandwidth in nm); CFP emission: 483/32; YFP (FRET) emission: 542/27; GFP excitation: 472/30; GFP emission: 520/35. To minimize photobleaching, a neutral density (ND) 64 filter (∼1.5% transmittance) was used during imaging experiments. Time–lapse images were acquired with a 40× (0.75 numerical aperture) objective at an interval of 2 min or 10 min. FRET images for GTPase activity was generated with NIS-Elements software (Nikon) by computing an emission ratio of YFP/CFP for individual cells. The FRET ratio images were scaled according to the color bar. The GFP images for TCF/LEF activity and β-catenin localization were background-subtracted and the fluorescence intensity was averaged over the whole cell area or over the nucleus by using NIS-Elements software (Nikon). The GFP images of β-catenin were scaled according to the color bar as described previously [25].

2.7. Statistical analysis

All statistical data were analyzed using Prism 5 software (GraphPad Software, La Jolla, CA, USA) and presented as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Dunnett's test was used for multiple comparisons with a control group. Student's *t*-test was used to compare the difference between two groups.

3. Results

3.1. Fluid flow-induced shear stress increases TCF/LEF activity

To study the potential role of Rho family GTPases in TCF/LEF activity under flow-induced shear stress, we first evaluated the effect of shear stress in regulating TCF/LEF activity. We transfected MC3T3-E1 cells with a TCF/LEF reporter and conducted real-time monitoring of its activity while applying oscillatory (1 Hz) shear stress of 10 dynes/cm^2, which is known to be within the physiological range subjected in bone [26]. In response to shear stress, the