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Focal adhesion kinase mediates β-catenin signaling in periodontal ligament cells

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ABSTRACT

Periodontal ligament (PDL) cells convert the orthodontic forces into biological responses by secreting signaling molecules to induce modeling of alveolar bone and tooth movement. Beta-catenin pathway is activated in response to mechanical loading in PDL cells. The upstream signaling pathways activated by mechanical loading resulting in the activation of β-catenin pathway through Wnt-independent mechanism remains to be characterized. We hypothesized that mechanical loading induces activation of β-catenin signaling by mechanisms that dependent on focal adhesion kinase (FAK) and nitric oxide (NO). We found that mechanical or pharmacological activation of β-catenin signaling in PDL cells upregulated the expression of β-catenin target genes. Pre-treatment of PDL cells with FAK inhibitor-14 prior to mechanical loading abolished the mechanical loading-induced phosphorylation of Akt and dephosphorylation of β-catenin. PDL cells pre-treated with NO donor or NO inhibitor and subjected to mechanical loading. Western blot analysis showed that the mechanical loading or pre-treatment with NO donor increased the levels of dephosphorylated β-catenin, pAkt, and pGSK-3β. Pre-treatment with NO inhibitor blocked the mechanical loading-induced phosphorylation of Akt and dephosphorylation of β-catenin. These data indicate that mechanical loading-induced β-catenin stabilization in PDL cells involves phosphorylation of Akt by two parallel pathways requiring FAK and NO.

1. Introduction

Modeling of alveolar bone around a tooth subjected to orthodontic force is essential for orthodontic tooth movement. Periodontal ligament cells (PDL) are subjected to mechanical loading during orthodontic mechanotherapy. PDL cells are generally thought to be the transducers of forces applied to teeth to induce their movement. PDL cells convert the mechanical stimulus into biological response by secreting signaling molecules that modulate the number and behavior of osteoblasts and osteoclasts [1,2]. However, the precise molecular mechanism underlying transduction of physical stimulus to signaling molecules to induce alveolar bone modeling and tooth movement remains to be fully investigated.

In PDL cells, a number of signaling molecules such as NO [3,4] and ATP [5,6] are released immediately following an episode of mechanical loading. Expression of cyclooxygenase-2 (COX-2) (enzyme responsible for prostaglandin synthesis) and prostaglandin E_2 (PGE_2) are upregulated in response to mechanical stimulation of PDL cells [1,7,8]. In osteoblasts and osteocytes, these molecules are associated with strain-related downstream events such as activation of β-catenin signaling [9,10]. COX-2 which regulates PGE_2 synthesis is a β-catenin target gene in osteoblasts [11–13]. Mechanical loading induces nuclear translocation of β-catenin and activation of β-catenin signaling in PDL cells through Wnt-independent pathway [14]. However, the upstream signaling pathways activated by mechanical loading resulting in the activation of β-catenin pathway through a lipoprotein receptor-related protein 5(LRP5)-independent process remains to be characterized.

Focal adhesion kinase (FAK) plays a key role in converting mechanical signals into a biological response through the activation of cytoplasmic signaling molecules [15]. The finding that FAK regulates PGE_2 synthesis via transcriptional control of COX-2 in PDL cells subjected to mechanical stimulation [16], indicate a potential role for FAK in mechanical loading-induced activation of β-catenin signaling.

Nitric oxide (NO) produced by osteocytes and osteoblasts in response to mechanical loading is associated with transduction of mechanical stimulus into a biological response in bone [17,18]. Recent studies in osteoblasts and osteocytes have demonstrated that mechanical loading-induced activation of β-catenin signaling is dependent on NO production [10,19]. Mechanical stimulus has

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been shown to induce NO production in PDL cells [3,4,20]. However it is unknown whether strain-mediated activation of β-catenin signaling in PDL cells is dependent on NO production.

To elucidate the signaling pathways that are activated immediately following an episode of strain in PDL cells; we sought to examine the role of FAK and NO in mechanical loading-induced β-catenin signaling in PDL cells. We hypothesized that mechanical loading induces dephosphorylation of β-catenin and activation of β-catenin signaling by mechanisms that dependent on FAK and NO.

2. Materials and methods

2.1. Cell culture and reagents

Primary human PDL cells were obtained from ScienCell (Carlsbad, CA, USA) and cultured in Minimum Essential Medium-alpha modification (CellGro, Manassas, VA, USA) supplemented with antibiotics and 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Cells between third and eighth passages were used in experiments. LiCl (Fluka Sigma Aldrich, St. Louis, MO, USA), which activates β-catenin signaling was used at a concentration of 40 mM. SNAP (Cayman Chemical, Ann Arbor, Michigan, USA), a nitric oxide donor was used at a concentration of 1 mM. L-NAME (Cayman Chemical), a nitric oxide inhibitor was used at concentration of 1.5 mM. FAK inhibitor 14 (1, 2, 4, 5-Benzenetetramine tetrahydrochloride) (Tocris Bioscience, Ellisville, MO, USA) is a selective focal adhesion kinase (FAK) inhibitor that prevents FAK autophosphorylation was used at a concentration of 100 mM. LY294002 (Cayman Chemical), a PI3 kinase inhibitor was used at a concentration of 10 μM.

2.2. Mechanical loading

PDL cells were plated in six-well plates at a density of 50,000 cells/cm^2 on the day before the experiment and mechanical loading was applied by placing 25 mm diameter cover glasses and/or customized glass cylinders over the cells as described previously (Fig. 1) [1]. Force magnitude was adjusted by adding or removing lead granules. PDL cells were subjected to 0.2 g/cm^2, 2.2 g/cm^2, and 5 g/cm^2 compressive force.

2.3. Protein extraction

Whole cells lysates were prepared with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Protein concentration was estimated by BCA Protein Assay (Thermo Fisher, Rockford, IL, USA) with bovine serum albumin as standard.

2.4. Western blot analysis

Equal amounts of protein were run on polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were incubated with primary antibodies overnight. On the following day, the membranes were incubated with Alexa Fluor 680 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA), 1:5000 dilution in PBST for 1 h. Proteins were detected with Li-Cor Odyssey System (Li-Cor, Lincoln, NE, USA). The primary antibodies used were against: Active β-catenin (Clone 8E7; Millipore, Billerica, MA,USA), Total β-catenin (gift from Dr. James Wahl, University of Nebraska Medical Center), pGSK3β (Serine^9; Cell Signaling Technology), Total GSK3β (Cell Signaling Technology), Akt (Cell Signaling Technology), pAkt (Serine^{473}; Cell Signaling Technology), COX-2 (Cayman Chemical), FAK (Cell Signaling Technology), phosphorylated FAK (Ser^{722} Santa Cruz Biotechnology, Inc. Dallas, TX, USA), Cyclin D1 (Cell Signaling Technology) and GAPDH (Novus Biologicals, Littleton, CO, USA).

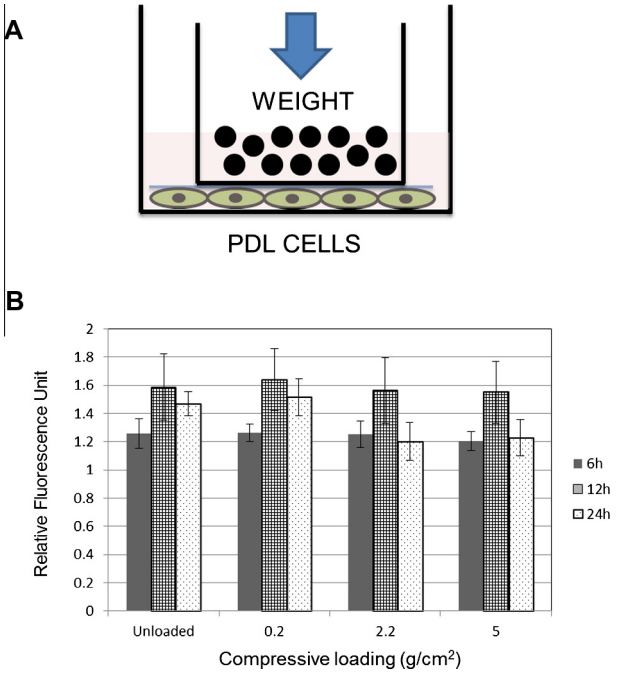

Fig. 1. Method of application of compressive loading and viability of PDL cells subjected to compressive loading. (A) Schematic diagram of the method used to apply mechanical loading. PDL cells were pre-cultured in 6-well plates containing culture medium. Compressive force was applied by placing a customized glass cylinder containing lead granules on top of the PDL cells. Force magnitude was changed by adding or reducing lead granules. PDL cells were subjected to 2.2 g/cm^2 and 5 g/cm^2. Controls: Cells plated on cover glass and place upside-down without any load (0.2 g/cm^2) and cells plated on 6-well plate without any load. (B) Periodontal ligament cell viability/proliferation as measured by Alamar blue assay. Error bars represent standard deviation. (For interpretation of color in Fig. 1, the reader is referred to the web version of this article.)

2.5. Enzyme-linked immunosorbent assays (ELISA) for PGE_2

Levels of PGE_2 released in the conditioned media were determined using a Prostaglandin E_2 EIA Kit (Cayman Chemical) according to manufacturer's instructions. The cells were collected and total protein was quantified to normalize the concentration of PGE_2 in the supernatant. Conditioned media of 3 independent experiments were assayed in duplicate.

2.6. Alamar blue assay

Periodontal ligament cells were subjected to 0.2 g/cm^2, 2.2 g/cm^2, and 5 g/cm^2, as described previously. Viability and proliferation of periodontal ligament cells were determined by alamar blue assay. Fresh media containing 10% Alamar Blue dye (Invitrogen, Carlsbad, CA) was added to the cells subjected to mechanical loading. After 3-h incubation, a small sample of the medium was collected and the cell number was determined by measuring the fluorescence intensity of the dye in a fluorescent spectrophotometer (Elx808, BioTek Instruments, Winooski, VT, USA). The excitation wavelength was 544 nm and the emission wavelength was 590 nm. Results were reported in arbitrary absorption unit. The fluorescence intensity of the sample media is directly related to the cell viability/ proliferation.