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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).

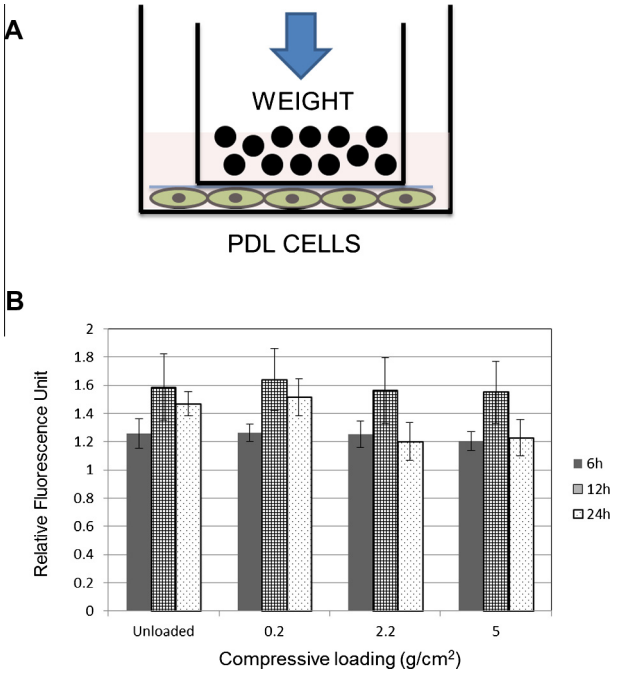

Fig. 1. Method of application of compressive loading and viability of PDL cells subjected to compressive loading. (A) Schematic diagram of the method used to apply mechanical loading. PDL cells were pre-cultured in 6-well plates containing culture medium. Compressive force was applied by placing a customized 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.

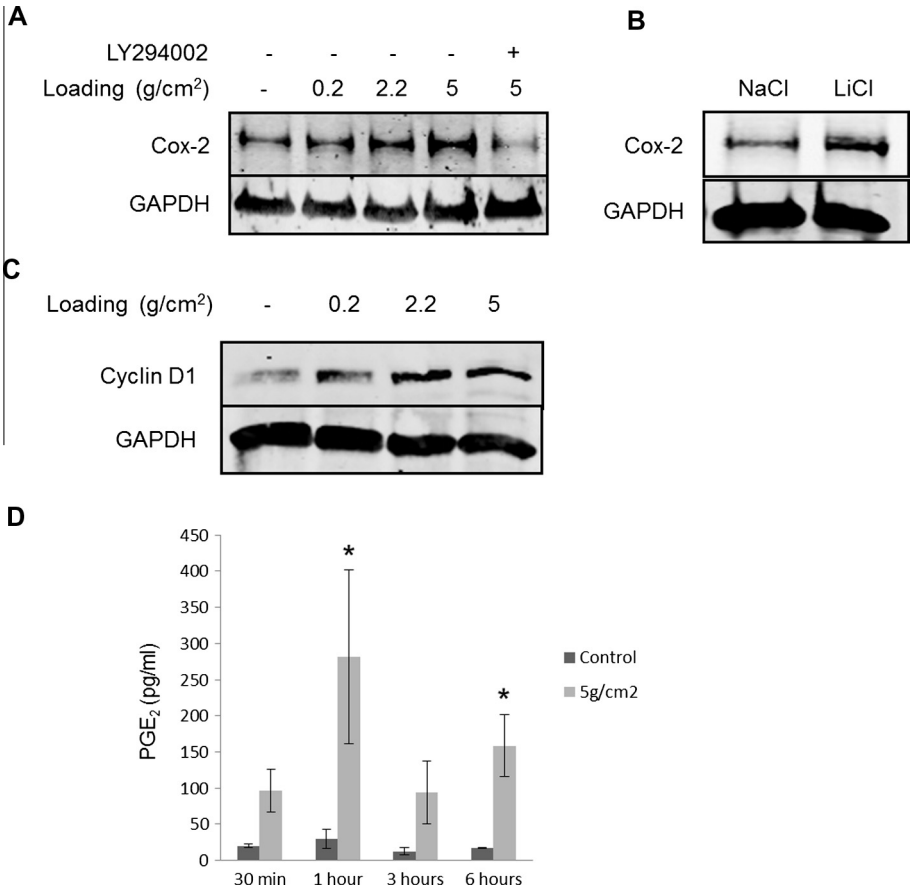

Fig. 2. Mechanical loading upregulates expression of β-catenin target genes and PGE_2 in PDL cells. PDL cells were subjected to 0.2 g/cm², 2.2 g/cm², and 5 g/cm² of mechanical loading for 6 h. (A) Western blot analysis of COX-2 expression in periodontal ligament cells subjected to mechanical loading and treated with LY294002 (10 μM). (B) Western blot analysis of COX-2 expression in PDL cells treated with LiCl (40 mM) compared with NaCl treatment. (C) Western blot analysis of Cyclin D1 expression in periodontal ligament cells subjected to mechanical loading. (D) ELISA determination of time-dependent changes in the PGE_2 production in PDL cells subjected to mechanical loading.

2.7. NO Measurement

Nitric Oxide is a very unstable gas, which is rapidly converted into breakdown products, such as nitrite. Levels of nitrite present in cell-conditioned media were quantified using Griess Reagent System. This assay relies on a diazotization reaction which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Sulfanilamide and NED solutions were purchased from Ricca Chemical Company (Arlington, TX, USA). 0.1 M Sodium Nitrate was used as standard. Conditioned media of three independent experiments were assayed for nitrite concentration in duplicates. The absorbance was measured at 540 nm.

2.8. Statistical analyses

Analyses among multiple groups were determined by Analysis of Variance (ANOVA) and analyses between 2 groups were determined by Student's *t*-test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Compressive loading upregulated the expression of COX-2 and Cyclin D1 in PDL cells

To determine whether static compressive loading of PDL cells affect their viability, alamar blue assay was performed. There was no significant difference in the viability between periodontal ligament cells subjected to static compressive loading and unloaded controls (Fig. 1). Mechanical loading has been shown to upregulate β-catenin genes such as COX-2 and Cyclin D1 in osteoblasts [13]. PDL cells were subjected to mechanical loading for 6 h or treated with LiCl for 60 min. Expression levels of COX-2 and Cyclin D1 were determined by Western immunoblotting. Expression of COX-2 and Cyclin D1 were upregulated in a force-magnitude dependent manner in PDL cells subjected to mechanical loading (Fig. 2A and C). Western blots showed an increase in COX-2 in cells treated with LiCl (Fig. 2B). LiCl is an inhibitor of GSK-3β (Glycogen Synthase Kinase 3 beta) activity and activates β-catenin signaling by phosphorylating at Ser^9[19]. COX-2 expression was inhibited.when PDL cells were pre-treated with LY294002, suggesting an involvement of PI3K/Akt pathway in the mechanical loading-induced upregulation of COX-2 expression (Fig. 2A).

3.2. Static compressive loading induced the production of PGE_2 from PDL cells

ELISA assays were used to determine the effect of static compressive loading on PGE_2 production and release. The production of PGE_2 was increased in a time-dependent manner and PDL cells subjected to loading for 1 h exhibited a significantly increased PGE_2 production compared with that of control PDL cells (Fig. 2D).

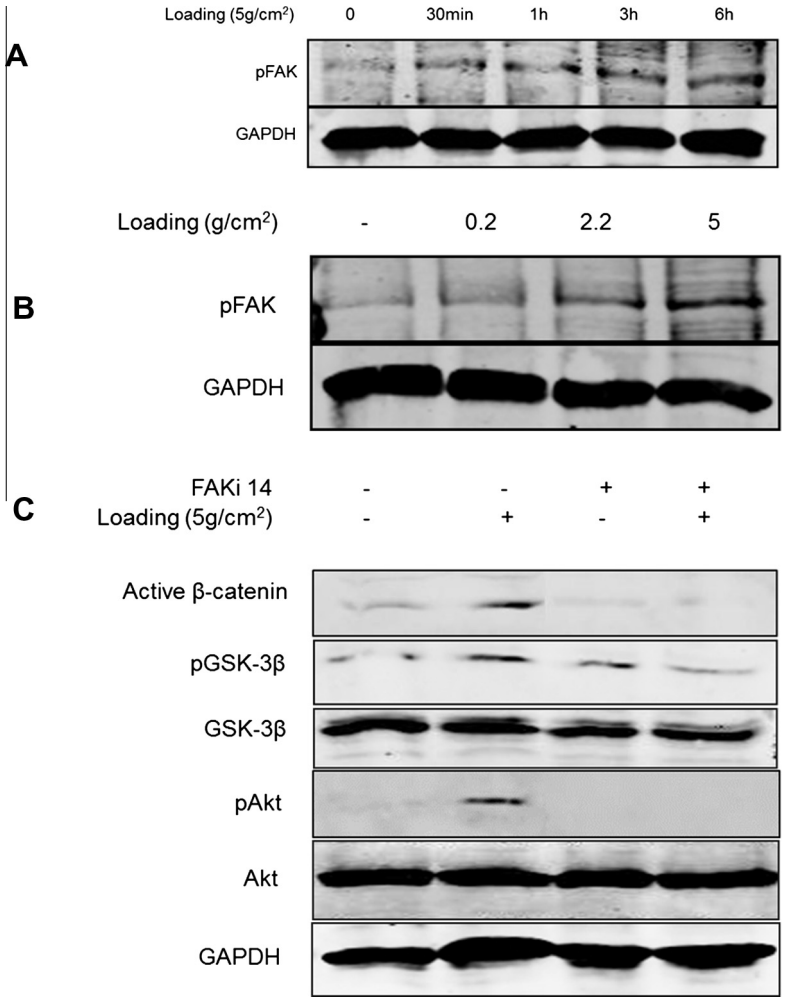

Fig. 3. Mechanical loading-induced dephosphorylation of β-catenin in periodontal ligament cells is mediated by FAK. (A and B) Periodontal ligament cells were subjected to 0.2 g/cm^2, 2.2 g/cm^2, and 5 g/cm^2 of mechanical loading for 6 h. Western blot analysis of phosphorylated FAK (at Ser^{722}) expression in periodontal ligament cells subjected to mechanical loading. GAPDH expression was used as a loading control. (C) Periodontal ligament cells were pre-treated with FAK inhibitor-14 (100 μM) for 1 h and subjected to 5 g/cm^2 of mechanical loading for 6 h. Western blot analysis showed that FAKi14 inhibited the mechanical loading-induced dephosphorylation of β-catenin and phosphorylation of Akt and GSK-3β. Levels of total β-catenin, total Akt, and total GSK-3β remained constant.

3.3. Compressive loading-induced activation of the β-catenin pathway is mediated by FAK in PDL cells

To determine whether mechanical loading induces phosphorylation, PDL cells were subjected to 0.2, 2.2, and 5 g/cm^2 mechanical loading for 6 h and the levels of FAK phosphorylated at Ser^{722} was determined by Western immunoblotting. In PDL cells, mechanical loading induced the phosphorylation of FAK in a time and force magnitude-dependent manner (Fig. 3A and B).

To assess whether the mechanical loading-induced dephosphorylation of β-catenin and consequent activation of β-catenin signaling pathway are due to activation of FAK, PDL cells were pre-treated with FAK inhibitor-14 for 1 h and subjected to mechanical loading for 6 h. Pre-treatment of periodontal ligament cells with FAK inhibitor-14 prior to mechanical loading experiments abolished the mechanical loading-induced phosphorylation of Akt and dephosphorylation of β-catenin. These findings suggest that β-catenin stabilization and activation of β-catenin pathway in response to mechanical loading occurs by FAK-mediated activation of PI3K/Akt pathway (Fig. 3C).

3.4. Activation of β-catenin signaling in periodontal ligament cells is mediated by nitric oxide (NO)

Nitric oxide is an early signaling molecule that plays a role in the mechanotransductive events in osteoblasts and osteocytes (Turner et al., 1995; Santos et al., 2010). To determine whether mechanical loading induced a similar NO-mediated signaling in PDL cells, NO release into the medium from PDL cells was measured after application of 5 g/cm^2 static compressive loading for 0.25, 0.5, 1, and 2 h. The NO measurement was performed using the Griess Reagent System. Mechanical loading significantly increased NO production at 30 min in PDL cells (Fig. 4A).

To determine whether mechanical loading-induced NO production involved in activation of β-catenin signaling, PDL cells were subjected to mechanical loading in the presence of NO inhibitor (L-NAME) or NO donor (SNAP). The mechanical loading-induced dephosphorylation of β-catenin was abolished in the presence of L-Name, suggesting that mechanical loading-induced NO production is involved in the activation of β-catenin signaling in PDL cells (Fig. 4B).

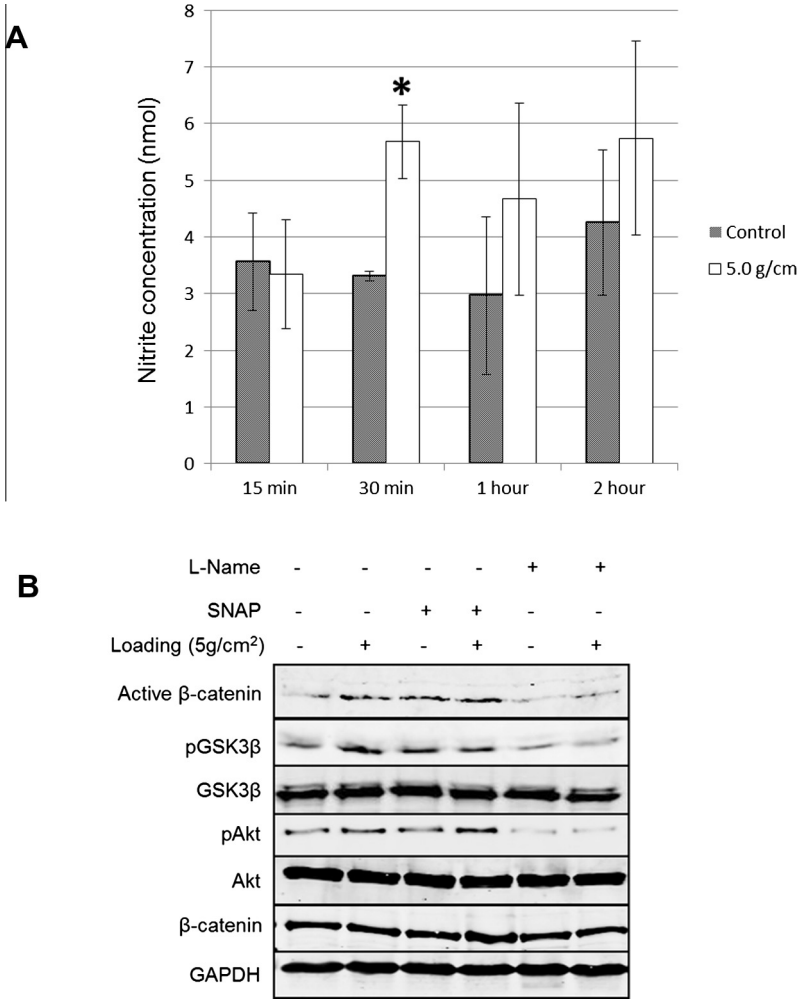

Fig. 4. Mechanical loading-induced activation of β-catenin signaling in periodontal ligament cells is mediated by Nitric Oxide. (A) Periodontal ligament cells were subjected to 5 g/cm^2 of mechanical loading for 0.2, 0.5, 1 and 2 h. The conditioned media of the cell cultures were collected to estimate nitric oxide production. NO released into the conditioned medium was measured using Griess Reagent System. Mean values are shown ± S.E.M., $n = 3$. $^*p < 0.05$. (B) To determine whether mechanical loading-induced NO production mediates activation of β-catenin signaling, PDL cells were pre-treated with SNAP (NO donor) (1 mM) or L-NAME (NO inhibitor) (1.5 mM) for 1 h and subjected to 5 g/cm^2 of mechanical loading for 6 h. 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 dephosphorylation of β-catenin as well as phosphorylation of Akt and GSK-3β. Levels of total β-catenin, total Akt, and total GSK-3β remained constant.

4. Discussion

PDL cells translate mechanical signals into biochemical responses resulting in orthodontic tooth movement. Mechanical loading is known to upregulate the expression of COX-2 and PGE_2 in PDL cells [1,7,8]. COX-2 is a direct target gene of β-catenin signaling and activation of β-catenin signaling has been shown to up-regulate Cox-2 expression in multiple cell types [12,21–23]. In this study for the first time, we show that pharmacological activation of β-catenin signaling enhances the expression of COX-2 in PDL cells.

We have previously shown that mechanical loading induces activation of β-catenin pathway in PDL cells through a Wnt-independent process [14]. Activation of β-catenin independent of signaling through Wnt ligands is mediated by phosphorylation of glycogen synthase kinase 3β (GSK3β) by Akt downstream signaling [24]. Mechanical loading can activate Akt signaling in multiple cell types including PDL cells [14,25]. The main goal of the present study was to understand the upstream events which were initiated by mechanical loading that lead to phosphorylation of Akt and stabilization of β-catenin. Recently it has been established that strain-mediated activation of Akt in osteoblasts and osteocytes occurred through two parallel pathways involving NO and FAK [9,10]. Therefore, in our studies we focused on the roles of NO and FAK in strain-mediated activation of β-catenin signaling in PDL cells.

In this paper, we report two major novel findings: (i) β-catenin stabilization and activation of β-catenin pathway in response to mechanical loading occurs by FAK-mediated activation of PI3K/Akt pathway (ii) mechanical loading-induced NO production is involved in the activation of β-catenin signaling in PDL cells.

PDL cells increase Cox-2 expression and increase the release of PGE_2 in response to exposure to mechanical loading [1]. In PDL cells with disrupted FAK signaling, PGE_2, M-CSF, TNF-α and RANKL response to mechanical loading is impaired [16,26]. This indicates that FAK is essential for mechanotransduction in PDL cells. Mechanical loading induces phosphorylation of FAK in PDL cells [16,27]. FAK is a mechano-sensitive kinase associated with integrin signaling and play a role in intracellular conversion of mechanical into a biochemical signal in osteoblasts, osteocytes and PDL cells [9,10,15,27]. Therefore we hypothesized that strain-mediated activation of β-catenin signaling in PDL cells is dependent on FAK activation. Our results revealed Ser^{722} phosphorylation of FAK

suggesting activation of FAK domain. Western blotting revealed that inhibition of FAK results in decreased phosphorylated Akt indicating that strain-mediated activation of PI3K/Akt pathway occurs via FAK activation.

NO has been shown to play a role in orthodontic tooth movement [20,28–30]. Mechanical loading has been shown to induce NO production in PDL cells [3,4]. Release of nitric oxide (NO) is one of the early responses of osteoblasts and osteocytes subjected to mechanical loading. Strain-induced activation of β-catenin signaling in osteoblasts and osteocytes is mediated partially by NO [9,10]. Our studies demonstrated a similar mechanism in PDL cells. We have shown that inhibition of mechanical loading-induced production of NO with L-NAME abolished the dephosphorylation of β-catenin.

In conclusion, our work attempts to provide a mechanism for mechanical loading-induced β-catenin stabilization in PDL cells involving phosphorylation of Akt by two parallel pathways requiring FAK and NO.

Acknowledgments

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