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Early activation of the β-catenin pathway in osteocytes is mediated by nitric oxide, phosphatidyl inositol-3 kinase/Akt, and focal adhesion kinase

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ABSTRACT

Bone mechanotransduction is vital for skeletal integrity. Osteocytes are thought to be the cellular structures that sense physical forces and transform these signals into a biological response. The Wnt/β-catenin signaling pathway has been identified as one of the signaling pathways that is activated in response to mechanical loading, but the molecular events that lead to an activation of this pathway in osteocytes are not well understood. We assessed whether nitric oxide, focal adhesion kinase, and/or the phosphatidyl inositol-3 kinase/Akt signaling pathway mediate loading-induced β-catenin pathway activation in MLO-Y4 osteocytes. We found that mechanical stimulation by pulsating fluid flow (PFF, 0.7 ± 0.3 Pa, 5 Hz) for 30 min induced β-catenin stabilization and activation of the Wnt/β-catenin signaling pathway. The PFF-induced stabilization of β-catenin and activation of the β-catenin signaling pathway was abolished by adding focal kinase inhibitor FAK inhibitor-14 (50 μM), or phosphatidyl inositol-3 kinase inhibitor LY-294002 (50 μM). Addition of nitric oxide synthase inhibitor L-NAME (1.0 mM) also abolished PFF-induced stabilization of β-catenin. This suggests that mechanical loading activates the β-catenin signaling pathway by a mechanism involving nitric oxide, focal adhesion kinase, and the Akt signaling pathway. These data provide a framework for understanding the role of β-catenin in mechanical adaptation of bone.

Introduction

Bones are subjected to a variety of mechanical loadings during daily activity. Within physiological limits, bones adapt their mass and structure to the loading conditions in order to efficiently bear the prevailing mechanical loads [1]. Osteocytes are generally thought to be the bone cells that sense these variations in mechanical forces acting on bone [2–5].

Osteocytes are terminally differentiated bone cells that are distributed throughout the bone and connected to each other via cytoplasmatic processes that reside within canaliculi in the mineralized bone, thereby forming a network of connected cells. When bones are loaded, the resulting bone deformation causes the interstitial fluid present around the osteocytes to stream through the lacuno-canalicular network from regions under high pressure to regions under low pressure [2–4]. This flow of fluid is sensed by the osteocytes [2,4,6]. They convert the physical stimulus into a biological response by secreting signaling molecules that modulate the behavior of the bone forming cells, the osteoblasts, and/or the bone resorbing cells, the osteoclasts [7–10]. New signaling pathways have been identified as key players in the overall process of bone adaptation to mechanical loading. One of these signaling pathways is the β-catenin signaling pathway [11–13]. This pathway affects cellular function by regulating β-catenin levels and its subcellular localization [14–16]. In the absence of any stimulus, cellular β-catenin levels are controlled by a destruction complex that contains amongst others glycogen synthase kinase 3β (GSK-3β) [11–16]. Binding of a Wnt molecule to the cell surface receptor complex consisting of human low density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6) and frizzled transmembrane proteins leads to the phosphorylation of GSK-3β and inactivation of the destruction complex [14–17], followed by stabilization of intracellular β-catenin levels, accumulation of β-catenin in the cytoplasm, and translocation of β-catenin to the nucleus, where it initiates target gene activation [17–22].

Abbreviations: FAK, focal adhesion kinase; GSK-3β, glycogen synthase kinase 3β; L-NAME, *N*-ω-nitro-L-arginine-methyl ester; LRP5, human low density lipoprotein (LDL) receptor-related protein 5; NO, nitric oxide; PFF, pulsating fluid flow; PI3K, phosphatidyl inositol-3 kinase.

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Little and colleagues were the first to show that mechanical adaptation of bone involves the Wnt/β-catenin pathway, and specifically the LRP5 membrane receptor [12]. It has also been reported the mechanical stimulation increases Wnt secretion [13]. On the other hand, mechanical loading induces nuclear translocation of β-catenin in osteoblasts *in vitro* independent of Wnt production or signaling through the LRP5 receptor [18–20]. Loading-induced nuclear translocation of β-catenin independent of Wnt production is likely mediated by Akt [21], that in turn phosphorylates GSK-3β [18,19,22]. Akt activation is strictly dependent on the upstream kinase phosphatidyl inositol-3 kinase (PI3K), but Akt activity can be controlled in either a stimulus-dependent manner or a cell type-dependent manner [23–28]. Akt activation due to PI3K occurs through binding of growth factors to the receptor tirosine kinases or via integrins [23,28]. The integrin-mediated activation of Akt occurs by ligand-independent mediated cell adhesion or by PI3K activation through binding to focal adhesion kinase (FAK) [24]. Focal adhesions are prime candidate structures for converting extracellular mechanical signals such as fluid shear stress into a biological response through the activation of cytoplasmatic signaling molecules [29]. FAK is necessary for bone regeneration in adult mice [30], but no data is available on whether FAK is involved in the process leading to β-catenin stabilization in osteocytes after mechanical loading.

Nitric oxide (NO) is an early signaling molecule that mediates the anabolic response of bone to mechanical loading *in vivo* [9]. We have shown earlier that NO is likely involved in the cascade of events leading to the activation of the β-catenin signaling pathway after mechanical loading by pulsating fluid flow (PFF) in osteocytes [31]. However it is unknown whether PFF-induced NO production affects β-catenin stabilization.

We have reported earlier that mechanical loading activates the β-catenin pathway in osteocytes by up-regulating β-catenin target genes [31]. At early time points this up-regulation of β-catenin target genes is likely dependent on β-catenin stabilization but independent of Wnt production, while at later time points the β-catenin target gene up-regulation is caused by the expression of Wnts [31]. Not much data is available on which signaling pathways are activated by mechanical loading resulting in the activation of the β-catenin pathway independent of Wnt production [31]. It has been reported that fluid shear stress and prostaglandin E_2 activate β-catenin signaling in MLO-Y4 osteocytes and 2T3 osteoblasts through an increase in GSK-3β phosphorylation, which results in β-catenin stabilization and thus activation of the β-catenin signaling pathway [21]. However these studies did not elucidate which other molecules are involved in activation of the β-catenin pathway. We hypothesize that mechanical loading induces stabilization of β-catenin by a mechanism that involves NO, focal adhesion kinase FAK, and the PI3K/Akt signaling pathway.

Materials and methods

Bone cell culture. MLO-Y4 osteocytes were cultured in α-Modified Eagle's Medium (α-MEM, Gibco, Paisley, UK) supplemented with 5% fetal bovine serum (FBS, Gibco), 5% calf serum (CS, Gibco), penicillin (10 μg/ml, Sigma, St. Louis, MO, USA), streptomycin (10 μg/ml, Gibco), gentamycin (50 μg/ml, Gibco), and fungizone (50 μg/ml, Gibco). The MLO-Y4 cells were kindly provided by Dr. L.F. Bonewald (University of Missouri-Kansas City, USA). MLO-Y4 osteocytes were harvested after reaching subconfluency, and seeded at 2.75×10^5 cells per collagen-coated (rat tail collagen type 1, 0.15 mg/ml, Sigma) glass slide (5 cm^2), and incubated overnight to promote cell attachment.

Pulsating fluid flow (PFF). PFF was generated using a flow apparatus containing a parallel flow chamber as described earlier [7]. Fluid shear stress of 0.7 ± 0.3 Pa at 5 Hz was induced for 30 min on the monolayer of cells by circulating 13 ml of α-MEM medium containing 0.1% bovine serum albumin (BSA) plus supplements as described above, and passing this medium through the parallel flow chamber with a roller pump. Static control cultures were kept in a petri dish under similar conditions as the experimental cultures, i.e. at 37 °C in a humidified atmosphere of 5% CO_2 in air. For the experiments using L-NAME, Fak inhibitor-14, and LY-294002, MLO-Y4 cells were incubated for 1 h prior to fluid flow experiments with 50 μM PI3-kinase activity inhibitor LY-294002 [19] (Enzo Life sciences BVBA, Zandhoven, Belgium), 10 μM FAK activity inhibitor FAK inhibitor-14 [32] (Tocris Biosciences, Bristol, UK), or 1.0 mM nitric oxide synthase inhibitor *N*-ω-nitro-L-arginine-methyl ester [9] (L-NAME, Sigma). Medium samples were taken at 5, 10, 15, and 30 min of PFF or control treatment, and assayed for NO concentrations as described below. After 30 min of PFF or control treatment, cells were lysed for β-catenin quantification, analysis of Akt phosphorylation levels, and expression of β-catenin target genes.

Nitric oxide (NO). NO production was measured as nitrite (NO_2^-) accumulation in conditioned medium using Griess reagent, as described earlier [31].

β-Catenin quantification. To determine total β-catenin concentration present in cell lysates, a Total β-catenin Enzyme Immunometric Assay Kit (Assay Designs Inc., Ann Arbor, MI, USA) was used. Immediately after PFF treatment, MLO-Y4 cells were washed with PBS, and the cell lysate was collected with RIPA cell lysis buffer containing 0.5 μL/ml of protease inhibitor cocktail (Sigma) and 1 mM of phenylmethanesulfonyl fluoride (Sigma). β-Catenin quantification was performed according to the manufacturer's protocol.

Akt phosphorylation detection. For IR detection of phosphorylated and non-phosphorylated Akt, blots were incubated with Anti-Phospho-Protein Specific Antibody (CASE™ Kit for AKT S473, SABiosciences, Frederick, MD, USA), Anti-Pan-Protein Specific Antibody (CASE™ Kit for AKT S473, SABiosciences), and mouse monoclonal anti-β-actin (Sigma) primary antibodies overnight at 4 °C in TBS, 0.05% Tween-20 supplemented with 1% BSA as blocking buffer, followed by incubation with IR-labeled secondary antibodies (Li-COR Biosciences, Cambridge, UK) for 60 min. Blots were imaged with Odyssey IR imager (Li-COR Biosciences) in 700 and 800 nm channels respectively in a single scan at 169 μm resolution. For two-color detection, primary antibodies were from different types of host animal. Secondary antibodies were from the same host species.

Analysis of gene expression. Gene expression of β-catenin target genes connexin 43, CD44, cyclin D1, and c-jun was studied using real time PCR as described previously [31]. Gene expression values were normalized for mouse GAPDH.

Statistical analysis. Data were obtained from four independent experiments. For statistical analysis, PFF-treated-over-control ratios of total β-catenin concentrations and gene expression of β-catenin target genes were calculated. Differences between groups were tested with Student's two tailed *t*-test for paired comparison or with Student's two tailed *t*-test single group mean and compared to 1. Differences were considered significant if $p < 0.05$.

Results

To assess whether mechanical loading by PFF modulates β-catenin levels in MLO-Y4 osteocytes, cells were treated with PFF or kept under static culture conditions (control) (Fig. 1). Application of PFF for 30 min to MLO-Y4 osteocytes did not result in visible changes in cell shape or alignment of the cells in a particular orientation (data not shown). No cells were removed by the fluid flow treatment, as assessed by visually inspecting the cultures before

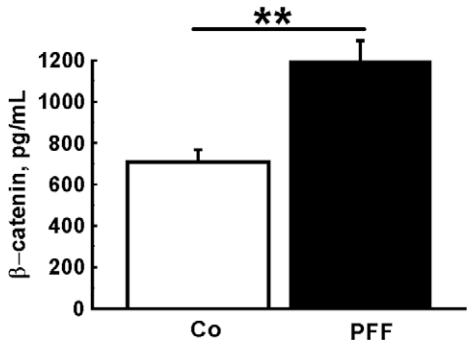

Fig. 1. PFF induces accumulation of total β-catenin in MLO-Y4 osteocytes. Thirty min of PFF stabilized total β-catenin concentration. Values are mean ± SEM of four independent cultures. PFF, pulsating fluid flow; Co, static control. **Significant effect of PFF, $p < 0.01$.

and after PFF treatment. Thirty minutes PFF treatment were sufficient to significantly up-regulate total β-catenin protein by 1.7-fold compared with static controls, indicating that fluid shear stress inhibits β-catenin degradation in MLO-Y4 osteocytes.

To test whether PFF-induced NO production modulates total β-catenin content, MLO-Y4 osteocytes were submitted to 30 min PFF in the presence of the NO inhibitor L-NAME (Fig. 2). PFF significantly increased NO production at 5 and 10 min by 2.3 and 1.9-fold, respectively (Fig. 2A). The PFF-induced NO production was abolished in the presence of L-NAME, which also inhibited the PFF-induced increase in total β-catenin concentration (Fig. 2B), suggesting that PFF-induced NO production is involved in the stabilization of total β-catenin.

To determine whether the mechanical loading-induced up-regulation of total β-catenin and consequent activation of the β-catenin signaling pathway is mediated by PI3K/Akt, MLO-Y4 osteocytes were submitted to 30 min PFF treatment in the presence of the PI3K inhibitor LY-294002 (Fig. 3). In MLO-Y4 osteocytes, 30 min PFF treatment increased the levels of phosphorylated Akt protein in comparison to static control cultures, but pre-treatment with LY-294002 for 1 h prior to PFF treatment abolished the PFF-induced effect on Akt phosphorylation (Fig. 3A). Pre-treatment of osteocytes for 1 h with LY-294002 followed by 30 min static incubation did not affect total β-catenin concentration, but LY-294002 decreased the PFF-induced up-regulation of total β-catenin by 1.7-fold (Fig. 3B). Thirty minutes PFF up-regulated the target genes CD44, connexin 43, cyclin D1, and c-fos by 1.7, 3.2, 2.4, and 1.9-fold, respectively. Pre-treatment with LY-294002 abolished the PFF-induced up-regulation of CD44, connexin 43, cyclin D1, and c-fos, indicating that PI3K/Akt is involved in stabilization of β-catenin and activation of the β-catenin pathway in response to PFF (Fig. 3).

To assess whether the PFF-induced changes in total β-catenin and consequent activation of the β-catenin signaling pathway are due to FAK activation of the PI3K/Akt pathway, MLO-Y4 osteocytes were submitted to 30 min PFF in the presence of FAK inhibitor-14 (Fig. 4). Pre-treatment of MLO-Y4 osteocytes with FAK inhibitor-14 for 1 h prior to PFF experiments, abolished the PFF-induced effect on Akt phosphorylation (Fig. 4A). Pre-treatment of MLO-Y4 osteocytes with FAK inhibitor-14 for 1 h followed by 30 min static incubation did not affect total β-catenin concentration, but FAK inhibitor-14 decreased the PFF-induced up-regulation of total β-catenin by 2.6-fold (Fig. 4B). Pre-treatment of osteocytes for 1 h with FAK inhibitor-14 followed by 30 min PFF down-regulated gene expression of CD44 by 1.3-fold but did not affect connexin 43, cyclin D1, and c-fos gene expression. Pre-treatment with FAK inhibitor-14 abolished the PFF-induced up-regulation of CD44, connexin 43, cyclin D1, and c-fos, suggesting that FAK promotes β-catenin stabilization and β-catenin pathway activation in response to PFF via activation of the PI3K/Akt pathway (Fig. 4).

Discussion

Activation of the Wnt/β-catenin signaling pathway has been shown to be essential for the adaptative response of bone to mechanical loading [11–13,18–20], but the role of the activation of this pathway in osteocytes upon mechanical stimulation is not fully understood. We and others have shown that mechanical loading induces β-catenin stabilization, followed by translocation of β-catenin to the nucleus, and expression of β-catenin target genes such as CD44, connexin 43, cyclin D1, and c-fos [11,19–21,31]. Mechanical loading immediately activates β-catenin independent of Wnt production, and in a later stage it activates β-catenin by stimulation of Wnt expression [11,19–21,31]. In this study we aimed to understand what upstream events resulted in the stabilization of β-catenin by mechanical loading independent of Wnt production in osteocytes. First, we showed that mechanical loading by PFF indeed results in stabilization of β-catenin and activation of the β-catenin pathway in osteocytes. We used the well established osteocyte cell line MLO-Y4, and measured total β-catenin increase as an indication of β-catenin stabilization, as well as gene expression of β-catenin target genes as an indication of activation of β-catenin signaling pathway. We found that short treatment by 30 min PFF induces an increase in total β-catenin concentration,

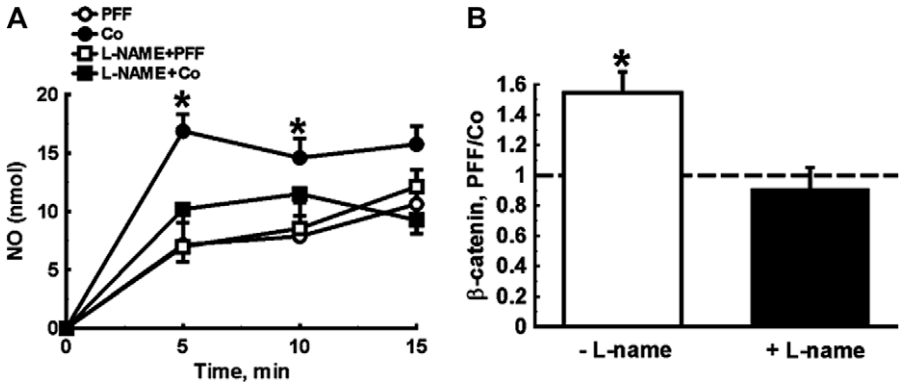

Fig. 2. PFF-induced accumulation of total β-catenin is mediated by NO in MLO-Y4 osteocytes. (A) PFF stimulated NO production within 5 min. The stimulatory effect is abolished in the presence of L-NAME. (B) Pre-incubation with L-NAME for 1 h followed by 30 min PFF abolished PFF-induced stabilization of total β-catenin concentration. Values are mean ± SEM of PFF-over-control ratios of four independent cultures. PFF, pulsating fluid flow; Co, static control; NO, nitric oxide; and L-Name, *N*-ω-nitro-L-arginine-methyl ester. *Significant effect of PFF, $p < 0.05$.

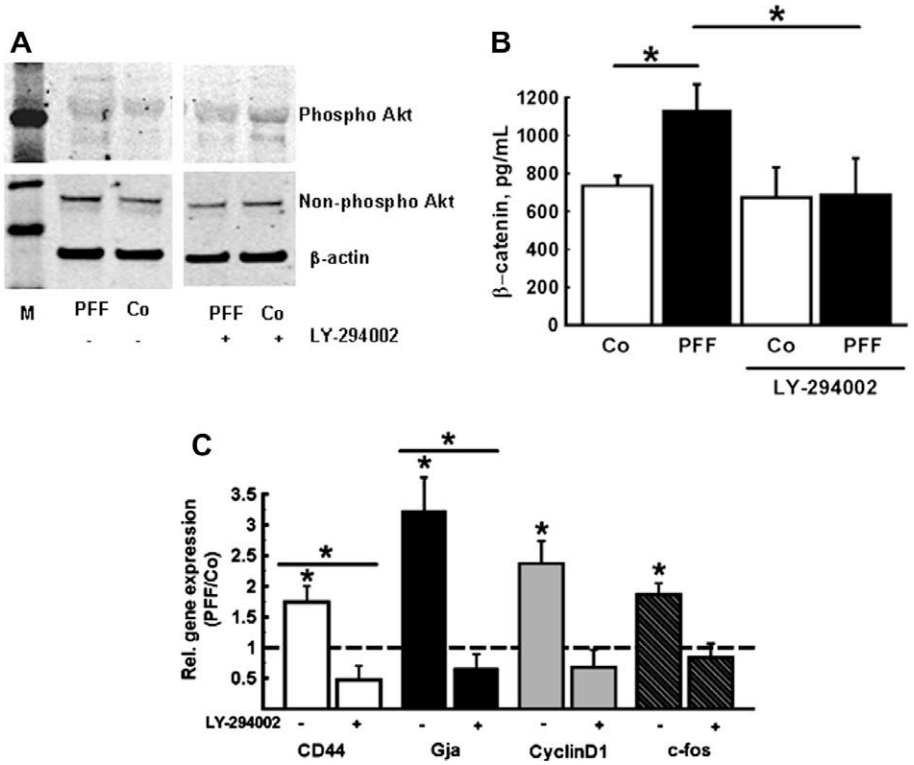

Fig. 3. PFF-induced activation of the β-catenin pathway is mediated by PI3K in MLO-Y4 osteocytes. (A) LY-294002 reduced the levels of phosphorylated Akt. (B) Pre-incubation with LY-294002 followed by 30 min PFF abolished PFF-induced stabilization of total β-catenin concentration. (C) Pre-incubation with LY-294002 followed by 30 min PFF abolished PFF-induced up-regulation of β-catenin target genes CD44, connexin 43, cyclinD1, and c-fos. Values are mean ± SEM of four independent cultures. PFF, pulsating fluid flow; Co, static control; LY-294002, PI3K inhibitor; and Gja, connexin 43. *Significant effect of PFF or LY-294002, $p < 0.05$.

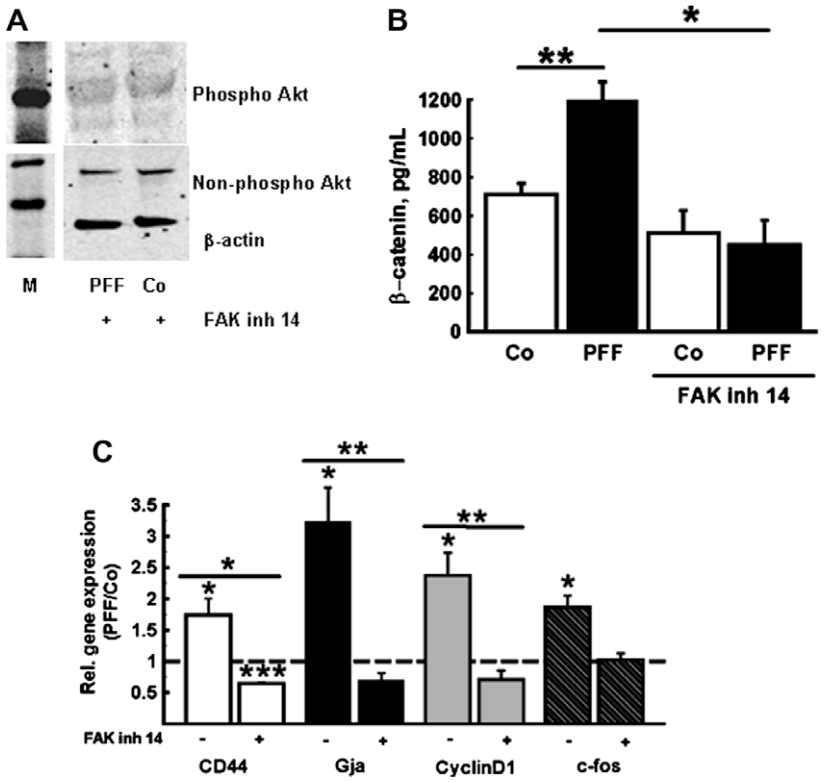

Fig. 4. PFF-induced activation of the β-catenin pathway is mediated by FAK in MLO-Y4 osteocytes. (A) FAK inhibitor-14 reduced the levels of phosphorylated Akt. (B) Pre-incubation with FAK inhibitor-14 followed by 30 min PFF abolished PFF-induced stabilization of total β-catenin concentration. (C) Pre-incubation with FAK inhibitor-14 followed by 30 min PFF abolished PFF-induced up-regulation of β-catenin target genes CD44, connexin43, cyclinD1, and c-fos. Values are mean ± SEM of four independent cultures. PFF, pulsating fluid flow; Co, static control; FAK inhibitor-14, Focal adhesion kinase inhibitor-14; and Gja, connexin 43. *Significant effect of PFF, $p < 0.05$, **$p < 0.01$; *Significant effect of FAK inhibitor 14, $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

and an up-regulation of the target genes CD44, connexin 43, cyclin D1, and c-fos indicating that PFF leads to activation of the β-catenin signaling pathway in MLO-Y4 osteocytes. Our results are in accordance with previously reported observations showing that fluid shear stress treatment of MLO-Y4 osteocytes results in β-catenin nuclear translocation and changes in expression of β-catenin target genes [21,31]. We have reported earlier that activation of the β-catenin signaling pathway through PFF-induced Wnt production occurs mainly at 1–3 h after ceasing 1 h PFF treatment in MLO-Y4 osteocytes [31]. Therefore the β-catenin stabilization observed immediately after ceasing 30 min PFF treatment likely occurred independent of Wnt binding to the LRP5/6 receptor. Our results are in accordance with other studies using the pre-osteoblastic cell line CIMC-4, showing that pre-treatment with the Wnt antagonist dickopf 1, which disrupts Wnt signaling by binding to the Wnt receptor LRP5, does not prevent strain-induced nuclear translocation of β-catenin and up-regulation of the β-catenin target genes WISP-2 and COX2 [19]. This suggests that mechanical stimulation directly affects β-catenin without involvement of Wnt molecules [19].

It has been proposed that any pathway that activates Akt could lead to stabilization of β-catenin through the phosphorylation of GSK-3β [21,33]. Therefore we focused on molecules that are involved in the bone response to mechanical loading and could lead to β-catenin stabilization, i.e. NO, PI3K, and FAK [9,23,24,28,29]. We have shown, for the first time, that inhibition of PFF-induced NO production with L-NAME results in inhibition of PFF-induced stabilization of β-catenin. NO is produced when L-arginine is converted to L-citruline in the presence of nitric oxide synthase (NOS) enzyme, molecular oxygen, NADPH, and other co-factors [34]. The mechanism by which NO could mediate β-catenin stabilization is unknown, but similar observations have been reported by others, i.e. NO derived from the inducible NO synthase (iNOS) positively correlates with up-regulation of the β-catenin target gene WISP in cancer cell lines [35]. Furthermore the NO donor DETA-NONOate elevates WISP-1 mRNA and protein expression through a β-catenin-dependent but Wnt-independent pathway in human colonic epithelial cell lines, suggesting a possible role for NO in stabilization of β-catenin [35]. Our observations do not elucidate the exact mechanism whereby PFF-induced NO production leads to β-catenin stabilization; this warrants further investigation.

We found that inhibition of PI3K with LY-294002 results in inhibition of PFF-induced stabilization of β-catenin and PFF-induced activation of the β-catenin pathway in MLO-Y4 osteocytes. This indicates that PI3K is involved in the signaling pathway that leads to stabilization of β-catenin in osteocytes in response to PFF. PI3K is required for the activation of Akt. Fluid shear stress-stimulated Akt phosphorylation and activation of down-stream Akt signaling pathways in human endothelial cells is mediated by PI3K [23]. Thus it is likely that PFF-induced stabilization of β-catenin occurs via PI3K phosphorylation of Akt. Our results are in agreement with the findings by Norvell et al. [20], showing that 1 h of fluid shear stress induces a transient but significant increase in phosphorylation of both GSK-3β and Akt in osteoblasts. In contrast, 2% strain for 15 min induces Akt activation independent of PI3K in the pre-osteoblastic cell line CIMC-4 [19]. These differences could be explained by the fact that osteoblasts and osteocytes do not respond similarly to mechanical loading, or by the differences in mechanical load regimes applied.

Akt activation might be controlled differently depending on the stimulus and the cell type [24]. The response of osteoblasts with disrupted FAK signaling to mechanical loading by oscillatory fluid flow is impaired, indicating that FAK is essential for mechanotransduction in osteoblasts [29]. It is unknown whether FAK has a similar role in osteocyte mechanotransduction. We found that PFF-induced stabilization of β-catenin and consequent activation of the β-catenin pathway was mediated by FAK in osteocytes. Chen et al. proposed that FAK activation by integrin binding is followed by binding of PI3K to phosphorylated Tyr 397 in FAK, which results in PI3K and Akt activation [28]. Activation of the PI3K/Akt signaling pathway leads to inhibition of GSK-3β and β-catenin stabilization. We show that inhibition of FAK results in a decrease in phosphorylated Akt, indicating that PFF-activation of PI3K/Akt likely occurs via FAK activation. Therefore it is possible that mechanical loading activates FAK in osteocytes in an integrin-dependent manner, and that this activation is followed by phosphorylation of Akt resulting in β-catenin stabilization and β-catenin pathway activation. Future research is needed to show that mechanical loading leads to integrin-dependent activation of FAK in osteocytes.

Conclusion

In conclusion, our results show that PFF induces β-catenin stabilization and activation of the β-catenin signaling pathway by a mechanism mediated by NO, FAK, and Akt in osteocytes. These data provide a framework for understanding the role of β-catenin in mechanical adaptation of bone. Recognition of the missing links in the mechanistic model that explains this activation will be the focus of future work.

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