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Fluid Shear Stress Induces β-Catenin Signaling in Osteoblasts

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Abstract. β-Catenin plays a dual role in cells: one at cell–cell junctions and one regulating gene transcription together with TCF (T-cell Factor) in the nucleus. Recently, a role for β-catenin in osteoblast differentiation and gene expression has begun to be elucidated. Herein we investigated the effects of fluid shear stress (FSS) on β-catenin signaling. FSS is a well-characterized anabolic stimulus for osteoblasts; however, the molecular mechanisms for the effects of this stimulation remain largely unknown. We found that 1 hour of laminar FSS (10 dynes/cm^2) induced translocation of β-catenin to the nucleus and activated a TCF-reporter gene. Analysis of upstream signals that may regulate β-catenin signaling activity revealed two potential mechanisms for increased β-catenin signaling. First, FSS induced a transient, but significant, increase in the phosphorylation of both glycogen synthase kinase 3β (GSK-3β) and Akt. Second, FSS reduced the levels of β-catenin associated with N-cadherin, suggesting that less sequestration of β-catenin by cadherins occurs in osteoblasts subjected to FSS. Functional analysts of potential genes regulated by β-catenin signaling in osteoblasts revealed two novel observations. First, endogenous, nuclear β-catenin purified from osteoblasts formed a complex with a TCF -binding element in the cyclooxygenase-2 promoter, and, second, overexpression of either a constitutively active β-catenin molecule or inhibition of GSK-3β activity increased basal cyclooxygenase-2 levels. Together, these data demonstrate for the first time that FSS modulates the activity of both GSK-3β and β-catenin and that these signaling molecules regulate cyclooxygenase-2 expression in osteoblasts.

Key words: COX-2 — GSK-3β — Shear stress — β-Catenin — Osteoblast

Introduction

Bone is a dynamic tissue that undergoes continuous remodeling throughout the life span, as it adapts to both its physiologic and its physical environments [1]. The remodeling process involves the coordinated activity of osteoclasts, which resorb existing bone, and osteoblasts, which secrete new bone [2]. Mechanical loading of bone tips this balance toward net accumulation of bone mass *in vivo* [3–6], whereas prolonged absences of mechanical stimulation, such as occur during periods of extended bedrest or weightlessness during space flight, result in net bone loss [7, 8]. Bone cells sense and respond to mechanical loads via stress-generated fluid flow inside the canalicular–lacunar networks and trabecular spaces within bone tissue [9–11]. In fact, fluid shear stress (FSS) alone has been shown to be a more potent stimulus for osteoblasts and osteocytes than mechanical strain [12, 13]. Our laboratory and those of others have demonstrated that subjecting osteoblasts to FSS increases expression of genes including c-fos and cyclooxygenase-2 (COX-2) [14–18]. Although it is clear that osteoblasts are sensitive to mechanical stimulation *in vivo* and *in vitro* the molecular pathways responsible for the transmission of FSS-induced signals in osteoblasts are not well defined. In this study, we investigated whether β-catenin plays a role in FSS-regulated signal transduction in osteoblasts.

β-Catenin performs dual functions in cells; it is both a component of cell–cell adherens junctions and a regulator of gene transcription. Normally, β-catenin that is not associated with cadherins is rapidly phosphorylated by glycogen synthase kinase 3β (GSK-3β) and targeted for degradation by a destruction complex made up of a group of proteins including GSK-3β, axin, and APC (Adenomatous Polyposis Coli) (reviewed in [19]). When GSK-3β is phosphorylated, it is inactivated and does not phosphorylate β-catenin, thereby resulting in a cytoplasmic pool of nonphosphorylated β-catenin that translocates to the nucleus. In the nucleus, β-catenin promotes gene transcription when it associates with members of the TCF/LEF (T-cell factor/Lymphocyte Enhancing Factor) family of transcription factors [20, 21]. Target genes of β-catenin/TCF/LEF include c-myc, cyclin D1, and potentially COX-2 [22–24].

Both GSK-3β and β-catenin have recently been shown to regulate gene expression and differentiation in

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osteoblasts. In 2002, Smith et al. [25] demonstrated that glucocorticoids inhibit cell-cycle progression in differentiating osteoblasts by increasing the activity of GSK-3β, suggesting a role for GSK-3β in osteoblast differentiation. Kahler and Westendorf [26] described a LEF-1 response element in the osteocalcin gene and found that LEF-1 and β-catenin repressed Runx2–induced activation of the osteocalcin 2 promoter in osteoblasts. Evidence is beginning to emerge that the osteopontin gene may also be regulated by β-catenin/TCF. Increasing levels of a signaling pool of β-catenin correlate with increased osteopontin levels during induction of an epithelial-to-mesenchymal shift [27], and there is also evidence that TCF may regulate osteopontin expression in mammary tumor cells [28, 29].

Of particular relevance to bone biology is the potential role of β-catenin in regulation of COX-2 expression. A role for cyclooxygenases and prostaglandins in mechanoadaptation of bone has been established. Blocking prostaglandin synthesis *in vivo* by using inhibitors of cyclooxygenase activity prior to loading inhibits new bone formation [30, 31]. TCF-like promoter elements have been reported to exist in the COX-2 promoter [24, 32, 33], and evidence exists that correlates increased levels of nuclear β-catenin/TCF with increased levels of COX-2 [24, 32–35].

In this study, we found that 1 hour of 10-dynes/cm^2 laminar FSS stimulation inactivates GSK-3β, potentially via Akt activation. Consistent with inactivation of GSK3–β, we also measured β-catenin nuclear translocation and increased TCF-reporter gene activity after cells were subjected to 1 hour of FSS. FSS also induced a decrease in N-cadherin/β-catenin complexes, suggesting potentially less sequestration of β-catenin by cadherins during FSS. Furthermore, either direct inhibition of GSK-3β activity or activation of β-catenin signaling in static culture increased COX-2 expression in osteoblasts. Together, these studies indicate that FSS modulates GSK-3β and β-catenin signaling and that these molecules may play a role in the regulation of COX-2 expression in osteoblasts.

Materials and Methods

Isolation of primary rat calvarial osteoblasts

Primary rat calvarial osteoblasts were isolated according to the protocol described previously [36, 37]. Calvaria from 10 to 13 neonatal rats (birth to day 2) were isolated aseptically and minced and digested consecutively for 5, 20, and 45 minutes at 37°C in 0.2% collagenase P/0.25% trypsin. Cells released in the second and third digests (20- and 45-minute digests) were pooled and cultured in minimal essential media (GIBCO; MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Cells were passaged at confluence by brief trypsinization with media changed every other day. Supplementation of media with 100 μg ascorbic acid and 10 mM β-glycerophosphate was used to confirm that these cells differentiated, as evidenced by patchy staining for alkaline phosphatase activity.

Cell culture and fluid shear stress

MC3T3-E1 osteoblasts were cultured in α-Mem, and primary rat calvarial cells and UMR 106.01 osteosarcoma cells were cultured in MEM containing 10% FCS (Fetal Calf Serum) and 1% penicillin/streptomycin. Prior to each flow experiment, the cells were passaged onto glass slides, grown for 24 hours, and then serum-starved in the appropriate media + 0.5% FCS and 1% penicillin/streptomycin for 48 hours. For all experiments, both static (tissue culture incubator) and flowed cells were incubated at 37°C with 5% CO_2 in 30 mL of the appropriate media containing 0.1% FCS. Laminar FSS experiments were performed in parallel-plate flow chambers using a recirculating flow loop system designed by Frangos et al. [38] and marketed by Cytodyne (San Diego, CA, USA).

TCF reporter gene assay

UMR 106.01 cells were switched to media containing 0.5% FCS and 1% penicillin/streptomycin and then transfected using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA). Topflash (wild type TCF) or Fopflash (mutant TCF) luciferase reporter plasmids (Upstate Biotechnology, Lake Placid, NY, USA) were cotransfected into UMR 106.01 cells with a *Renilla* luciferase marker plasmid (pRL-TK, Promega, Madison, WI, USA). At 48 hours posttransfection, cells were subjected to 1 hour of either static or FSS conditions as described previously. Cells were then incubated for 4 hours in 12 mL media + 0.1% FCS and 1% penicillin/streptomycin, after which the cells were processed for Dual Luciferase Assays (Promega). For LiCI experiments, transfected cells were treated with 40 mM LiCI (Sigma Chemical Co., St. Louis, MQ, USA) for 4 hours in 12 mL media + 0.1% FCS and 1% penicillin/streptomycin and then processed as described previously. Results were calculated by standardizing to *Renilla* luciferase counts and then subtracting the mutant (Fopflash) luciferase counts from the wild type (Topflash) counts.

Generation of mutant, β-catenin-expressing cells

The mutant β-catenin was cloned into a pcDNA3 expression vector (Invitrogen Corp., Carlsbad, CA, USA), and comprises a c-myc epitope tag and the last 4 of 5 potential serine and threonine GSK-3β phosphorylation sites in the *N*-terminus converted to alanine. This construct was kindly provided by Dr. A. Kowalczyk (Emory University). Multiple, 10-cm dishes of MC3T3-E1 cells were transfected with the mutant β-catenin, selected in 400 μg/mL G418 (Sigma Chemical Co.), and drug-resistant colonies were pooled for immunoblot analysis. For controls, MC3T3-E1 cells were transfected with empty pcDNA3 vector (Invitrogen Corp.) and selected in parallel.

Electrophoretic mobility shift assays (EMSA)

EMSAs were performed as previously described by Alvarez et al. [39]. MC3T3-E1 cell nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit from Pierce Biotechnology, Inc. (Rockford, IL, USA). Prior to use in EMSAs, nuclear extracts were dialyzed and concentrated into a buffer (pH 7.9) containing 120 mM KCl, 20 mM EDTA, and 25 mM Hepes using Slide-a-Lyzer Mini Dialysis Units from Pierce Biotechnology, Inc. for dialysis and Centricon centrifugal filters from Millipore Corporation (Bedford, MA, USA) for concentration. The 20-μL binding reactions included 75 mM KCl, 15% glycerol, 0.15 mM EDTA, 1.0 μg of poly (dI.dC), 0.1 mM dithiothreitol, 19 mM Hepes (pH 7.5), 0.0075% Nonidet P-40, 6 μg soluble nuclear protein, and 60K cpm of labeled probe. The sequence of the EMSA probe for TBE1 is 5′-AT-

GTTGTACTTTGATCCATGGTCA-3′ and for TBE2 is 5′-CCGCTTCCTTTGTCCATCAGAA-3′ based on COX-2 promoter sequence published by Tazawa et al. [40]. These oligos were chosen because they contained the published TCF consensus sequence CTTTG(A/T)(A/T) [41, 42]. Oligos were end-labeled, annealed, and purified by gel electrophoresis (8% TBE polyacrylamide gels, eluted overnight at 37°C, and concentrated). For supershift analysis, 2 μg of a monoclonal TCF-3/4 antibody from Upstate, Inc. (Lake Placid, NY, USA), or 1.5 μg of a monoclonal β-catenin antibody from BD Transduction Laboratories (San Diego, CA, USA) were used.

Immunofluorescence Microscopy

Immediately following FSS or static conditions, cells were fixed in ice-cold 4% paraformaldehyde and permeabilized in 0.2% triton/PBS. Primary antibodies: Anti-β-catenin antibody from Zymed (San Francisco, CA, USA), anti-c-myc antibody (9e10) against the c-myc epitope tag (Sigma Chemical Co). Secondary antibodies: anti-mouse Ig rhodamine-conjugated antibodies from Jackson Immunoresearch (West Grove, PA, USA). Images were recorded using an RT Color Spotdigital camera (Diagnostic Instruments, Sterling Heights, MI, USA) attached to an Optiphot-2 Nikon epifluorescent microscope using a 60X Nikon planapo objective (1.4 numerical aperture).

Immunoblots, Immunoprecipitations, and Densitometry

For immunoblots, whole cell lysates were collected in SDS sample buffer; equal protein (20 μg) was run on SDS-PAGE gels and transferred to nitrocellulose. Protein concentration of samples was determined using the amido black method [43]. Membranes were blocked by using 5% milk in PBS. Proteins were detected using the following antibodies: monoclonal anti-N-cadherin from BD Transduction Laboratories (San Diego, CA, USA), monoclonal anti-β-catenin from Zymed, monoclonal anti-Cadherin-11 from Zymed polyclonal anti-COX-2 from Cayman Chemical Co. (Ann Arbor, MI, USA), monoclonal anti-c-myc (9e10) from Sigma Chemical Co., monoclonal anti-GSK-3β from BD Transduction Laboratories, polyclonal anti-phospho-GSK3β-serine9 from Cell Signaling Technology (Beverly, MA, USA) and monoclonal anti-vinculin from BD Transduction Laboratories. The appropriate anti-mouse Ig or anti-rabbit Ig peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA). The antibody signal was detected using a chemiluminescence solution and imaged using a LAS-1000 plus luminescent image analyzer (Fujifilm, Sunnyvale, CA, USA) Densitometric analysis was performed by using Image Reader LAS-1000 software (Fujifilm).

Prior to COX-2 immunoblots in Figure 6A, cells were treated with 40 mM LiCI (Sigma Chemical Co.) or vehicle control (water) for 1 or 5 hours in 12 ml media + 0.1% FCS and 1% penicillin/streptomycin.

Immunoprecipitation using monoclonal anti-β-catenin from Zymed was performed under conditions that maintain protein complexes. Immunoprecipitation buffer contained 1% Triton-X-100, 145 mM NaCl, 10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2 mM EGTA, and 1 mM PMSF. Immune complexes were captured by using Protein A sepharose beads from Sigma Chemical Co. conjugated to goat-anti-mouse antibody from Jackson Immunoresearch Laboratories.

Statistical analysis

Statistical analysis was performed with the statistical package Statview©, version 5.0.1 (SAS Institute, Cary, NC).

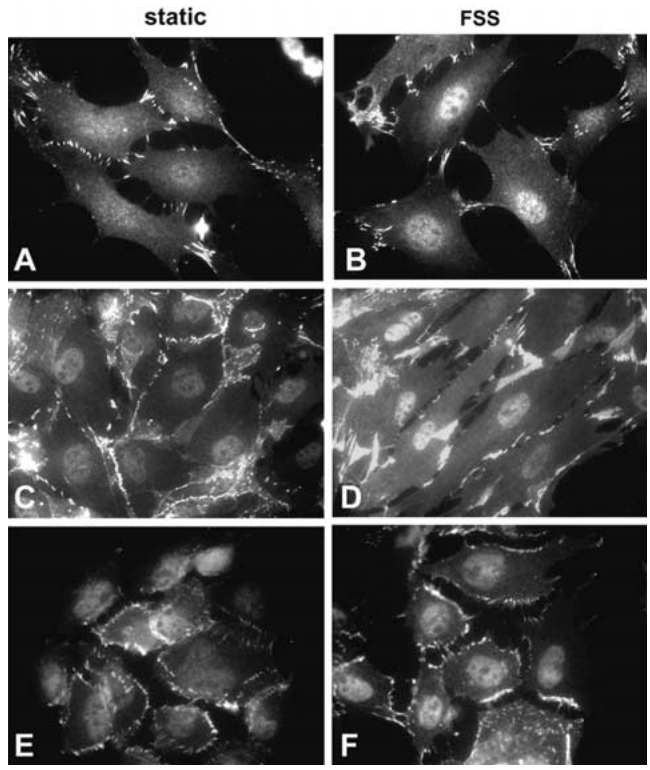

Fig. 1. FSS induces nuclear translocation of β-catenin. Immunofluorescence of osteoblasts using an antibody directed against β-catenin. Cells were subjected to 1 hour of static conditions (**A**, **C**, **E**) or 1 hour of 10-dynes/cm^2 laminar FSS (**B**, **D**, and **F**) and immediately fixed and processed for immunofluorescence. MC3T3-E1 osteoblasts (**A** and **B**), primary rat calvarial osteoblasts (**C** and **D**) and UMR 106.01 osteosarcoma cells (**E** and **F**) were tested for translocation of β-catenin. In cells subjected to FSS, there appeared to be more nuclear β-catenin than in cells held in static culture.

Results

Fluid Shear Stress Induces Nuclear Translocation of β-Catenin and Increases Signaling Through the TCF Transcriptional Pathway

To determine whether the subcellular distribution of β-catenin was affected by FSS, MC3T3-E1 osteoblasts (Figs. 1 A and B), primary rat calvarial osteoblasts (Figs. 1 C and D), and UMR 106.01 osteosarcoma cells (Figs. 1 E and F) were subjected to 1 hour of 10-dynes/cm^2 laminar FSS or 1 hour of static incubation. The value 10-dynes/cm^2 was chosen because it is within the range estimated to be physiologic in bone [44]. The subcellular localization of endogenous β-catenin was analyzed by using immunofluorescence microscopy. In cells held under static conditions, β-catenin localized primarily to sites of cell–cell attachment. There was also faint nuclear localization of β-catenin in static cells (Fig. 1 A, C, and E). However, in cells subjected to 1 hour of FSS there was increased localization of β-catenin to the nucleus (Fig 1 B, D, and F). After FSS, β-catenin was also still present at cell–cell interfaces. The nuclear

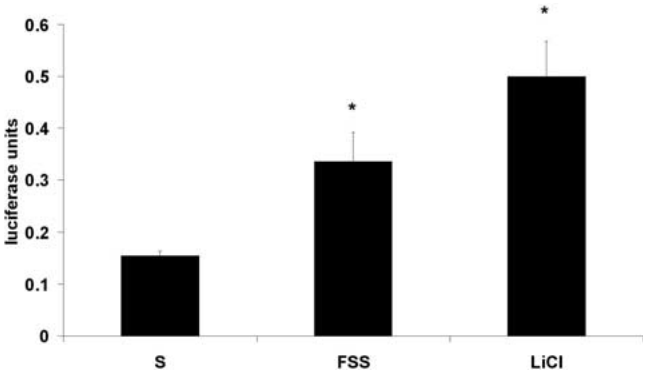

Fig. 2. TCF reporter gene activity is activated by FSS. UMR 106.01 cells were co-transfected with a wild type or mutant TCF luciferase reporter gene and *Renilla* luciferase plasmid. Posttransfection (48 hours) cells were subjected to 1 hour of 10 dynes/cm^2 laminar FSS or static conditions and then incubated for four additional hours. Luciferase activities were then assayed as described. Reporter activity was normalized by *Renilla* luciferase activity, and then mutant TCF luciferase activity was subtracted from wild type TCF luciferase activity. For a positive control, transfected cells were treated for four hours with 40 mM LiCl and processed as described. FSS induced a significant two-fold increase in TCF reporter gene activity and LiCl induced a significant three-fold increase in TCF reporter gene activity. S = static, FSS = fluid shear stress, LiCl = 40 mM LiCl treatment. $P < .05$, S vs. FSS and $P < .05$, S vs. LiCl, analysis of variance (ANOVA). $n = 9$.

localization was apparent after as little as 15 minutes of FSS and after as long as 5 hours of FSS (data not shown); however, the nuclear localization was most apparent after 1 hour. These time-points were chosen because it has been shown that osteoblasts may have a biphasic response to FSS. For example, there is an early peak of FSS-induced prostaglandin release at approximately 30 minutes and then a second, amplified release that occurs at approximately 2 hours and continues to increase for 4 to 6 hours [12, 45, 46].

Next, reporter gene assays were performed in UMR 106.01 cells because they transiently transfect at much higher efficiencies than either MC3T3-E1 cells or primary osteoblasts. Consistent with the FSS-induced visual translocation of β-catenin to the nucleus, FSS induced a significant, two-fold induction of TCF reporter gene activity compared to cells held in static culture (Fig. 2). This suggests that FSS induces translocation of β-catenin to the nucleus where it regulates gene transcription. Treatment with LiCl, a GSK-3 inhibitor also induced a significant, three-fold induction of TCF reporter gene activity.

Fluid Shear Stress Induces Activation of Akt and Inactivation of GSK-3β

To examine whether FSS modulates GSK-3β activity or the activity of the upstream GSK-3β regulator, Akt, MC3T3-E1 osteoblasts were subjected to 1 or 5 hours of 10-dynes/cm^2 laminar FSS. We chose MC3T3-E1 cells for these experiments and subsequent experiments because they show the most robust β-catenin nuclear translocation in response to FSS. After subjecting cells to 1 or 5 hours of FSS, the levels of tyrosine-473-phosphorylated Akt and serine-9-phosphorylated GSK-3β as well as total Akt and GSk-3 levels were determined by use of immunoblot analysis. The levels of phosphorylated and total Akt and GSK-3β as well as total Akt and GSK-3β levels were determined by densitometric analysis for each sample and a ratio of phosphorylated to total protein was calculated. After 1 hour of FSS, there was a significant, three-fold increase in the phosphorylation of tyrosine-473 of Akt (Fig. 3A) and a significant, doubling in the levels of phosphorylation of serine-9 of GSK-3β (Fig. 3B). After 5 hours of FSS, however, there was no longer a significant difference between static and flow samples for phosphorylation of either Akt or GSK-3β (Figs. 3A and B). Cells were also subjected to 15 minutes of FSS, but no change in phosphorylation of these proteins was detected (data not shown).

Fluid Shear Stress Decreases the Association Between β-Catenin and N-Cadherin but Does Not Alter Total Levels of N-Cadherin, Cadherin-11, or β-catenin

FSS may also promote β-catenin translocation by altering cadherin-β-catenin interactions or by modulating the steady-state levels of cadherins or β-catenin. We analyzed the steady-state levels of β-catenin and two osteoblast cadherins, N-cadherin and cadherin-11, in MC3T3-E1 cells held under static conditions or subjected to 1 hour of 10-dynes/cm^2 laminar FSS. Immunoblot analysis revealed that total levels of N-cadherin, cadherin-11, or β-catenin were not changed by FSS (Fig. 4A).

Next, we examined levels of cadherins associated with β-catenin in cells held under static conditions as compared to cells subjected to FSS to determine if FSS dissociates β-catenin from cadherins. β-Catenin was immunoprecipitated from cells held in static culture and from cells subjected to 1 hour of 10-dynes/cm^2 laminar FSS and the level of N-cadherin or cadherin-11 associated with the immunoprecipitated β-catenin was determined by immunoblot . Interestingly, in cells subjected to FSS, 50% less N-cadherin was associated with β-catenin as compared to static controls (Fig. 4B), whereas the amount of cadherin-11 associated with β-catenin was unaffected by FSS (Fig. 4B).

MC3T3-E1 Osteoblasts Contain a Nuclear Complex of β-Catenin and TCF That Interacts Directly with a TCF-binding Element in the Cyclooxygenase-2 Promoter

COX-2 is upregulated by FSS along a time-course similar to that of activation of β-catenin signaling [47]. COX-2 comprises a TCF-binding element that has not been demonstrated previously to bind endogenous TCF or β-

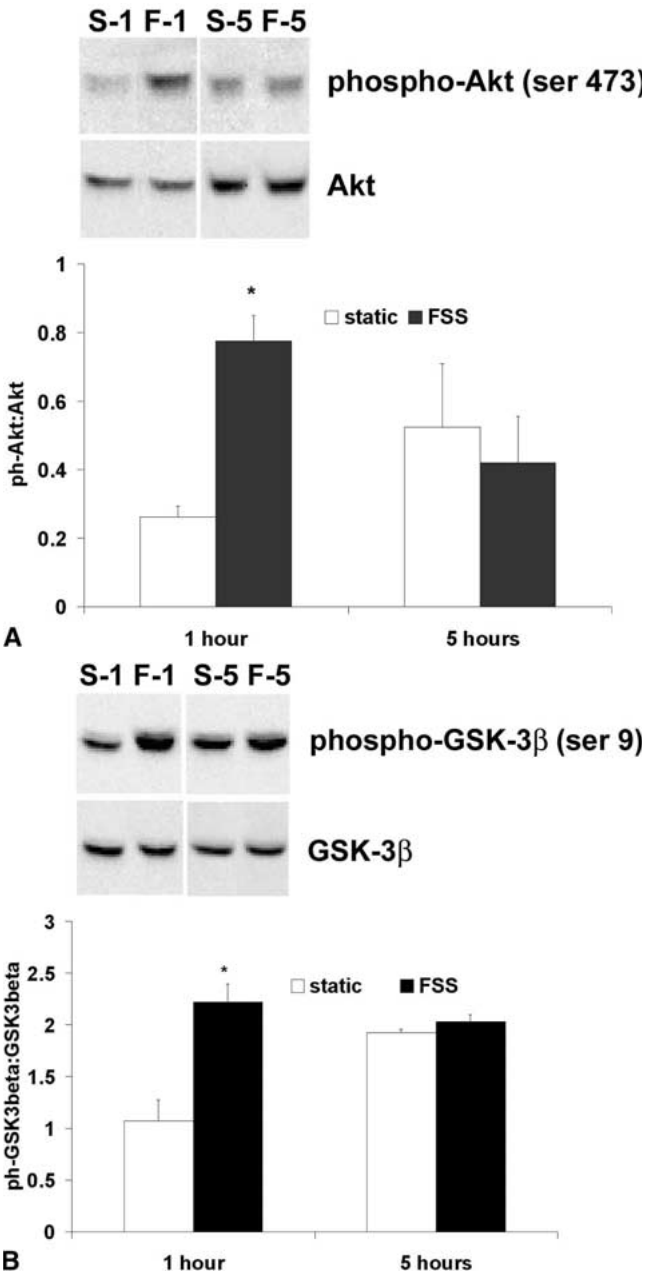

Fig. 3. FSS induces activation of Akt and inactivation of GSK-3β. MC3T3-E1 cells were subjected to 1 or 5 hours of 10-dynes/cm^2 laminar FSS, and the levels of phosphorylated Akt and GSK-3β, as well as to Akt and GSK-3β levels, were determined by immunoblot analysis. Equal protein (20 μg) from whole cell lysates was loaded onto SDS-PAGE gels and transferred to nitrocellulose. (**A**) 1 hour of FSS, but not 5 hours, induced a significant three-fold increase in Akt phosphorylation. Immunoblot analysis of the levels of phosphorylated Akt was performed by using an antibody specific for phospho-ser 473-Akt. The nitrocellulose was then stripped and re-blotted for total Akt protein. (**B**) 1 hour of FSS, but not 5 hours, induced a significant two-fold increase in serine-9-GSK-3β phosphorylation. Immunoblot analysis of the levels of phosphorylated GSK-3β was performed by using a phospho-ser 9-GSK-3β antibody. The nitrocellulose was then stripped and re-blotted for total GSK-3β protein. For quantification of both Akt and GSK-3β phosphorylation, densitometric analysis was performed on a minimum of three independent experiments and the ratio of phosphoprotein to total protein was determined. S = static and F or FSS = fluid shear stress. $P < .05$, F-1 vs. S-1 for both Akt and GSK-3β phosphorylation, Student's t test.

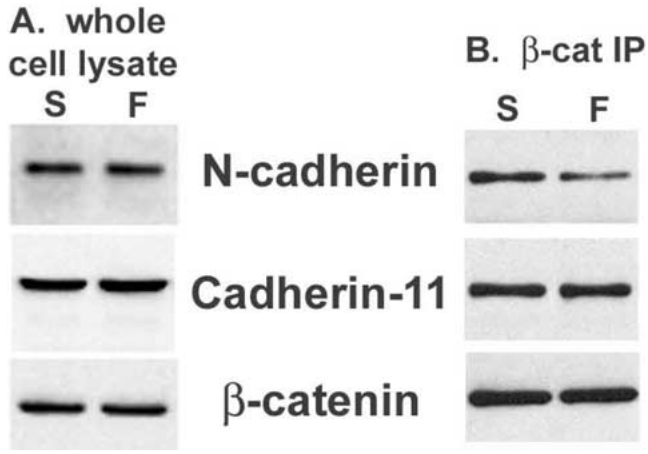

Fig. 4. FSS decreases the levels of N-cadherin associated with β-catenin by 50% but does not alter steady state levels of N-cadherin, cadherin 11, or β-catenin. (**A**) Immunoblot analysis of whole cell lysates of MC3T3-E1 cells subjected to 1 hour of static conditions or 1 hour of 10-dynes/cm^2 laminar FSS using antibodies directed against N-cadherin, cadherin 11, or β-catenin. Equal protein from whole cell lysates was loaded onto SDS-PAGE gels and transferred to nitrocellulose. FSS did not change the total levels of N-cadherin, cadherin-11, or β-catenin. Densitometric analysis of at least three experiments indicated no significant change in levels of N-cadherin, cadherin-11, or β-catenin. (**B**) Immunoprecipitation of β-catenin was performed from soluble fractions of equal protein from cells held for 1 hour in static culture or subjected to 1 hour of 10-dynes/cm^2 FSS. Immunoprecipitates were run on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted for N-cadherin, cadherin-11, and β-catenin. Densitometric analysis of bands revealed 50% less N-cadherin associated with β-catenin in cells subjected to FSS, but no change in cadherin-11/β-catenin complexes. S = static, F = fluid shear stress. Densitometric analysis of at least three experiments revealed significant changes in levels of N-cadherin associated with β-catenin: for N-cadherin/β-catenin, static vs. FSS. $P < .05$, Student t test. For Cadherin-11/β-catenin, $P = 0.3$, static vs. FSS, Student t test.

catenin from any cell type [24, 32, 33, 48]. To begin to elucidate the functional role of β-catenin in osteoblasts, we examined whether either of two putative TCF-binding elements (TBE) in the COX-2 promoter had the potential to interact with nuclear extracts from MC3T3-E1 osteoblasts. The first, TBE1, is located at-1079 to -1073, 1048 nucleotides upstream of the TATA box and the second, TBE2, is located at -867 to -871, 836 nucleotides upstream of the TATA box, as determined by the sequence published by Tazawa et al. [40]. A electrophoretic mobility shift assays, we examined whether either of these DNA elements interacted with nuclear fractions from osteoblasts. Nuclear extracts from osteoblasts induced a specific mobility shift using only the probe containing TBE1 (Fig. 5, lane 2). Nuclear extracts did not induce a mobility shift using the probe containing TBE2 (data not shown). The complexes containing TBE1 were specifically supershifted using antibodies directed against either TCF-3/4 or β-catenin (Fig. 5, lanes 4 and 6). A combination of the

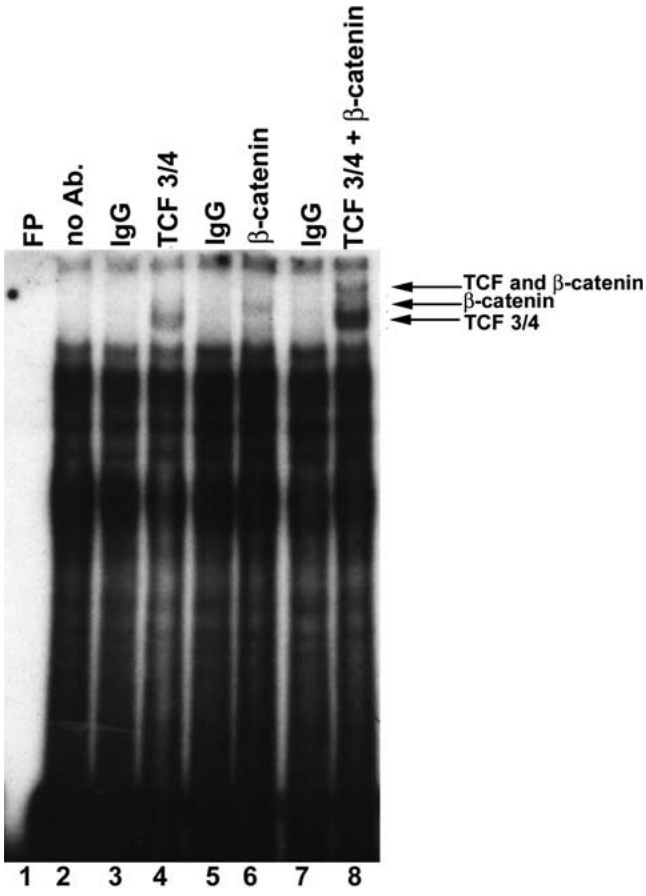

Fig. 5. β-Catenin and TCF interact directly with a TCF-binding element in the COX-2 promoter. EMSA was performed using a ^{32}P-labeled probe comprising promoter sequence from the COX-2 gene that contains a putative TCF-binding element. MC3T3-E1 cell nuclear extract was used as the source of protein. There was substantial complex formation as evidenced by the shift in lane 2. Supershift analysis was performed by using an antibody to TCF-3/4 (lane 4), an antibody to β-catenin (lane 6), both antibodies together (lane 8), or the appropriate amount of nonspecific antibody (lanes 3, 5, and 7). The arrows from top to bottom indicate the specific complexes formed by using the β-catenin and TCF-3/4 antibodies together, the β-catenin antibody, or the TCF-3/4 antibody, respectively. FP = free probe.

β-catenin and TCF-3/4 antibodies enhanced the TCF supershift (Fig. 5, lane 8). Furthermore, use of the combination of antibodies also induced a supershift of a higher molecular weight than either the β-catenin or TCF-3/4 antibody alone, suggesting the formation of a complex containing both β-catenin and TCF with TBE1 of the COX-2 promoter (Fig. 5, lane 8). This experiment was repeated three times. One representative experiment is shown.

Inhibition of GSK-3β Activity with LiCl or Increasing β-Catenin Signaling Increases Basal Cyclooxygenase-2 Protein Levels

To identify whether COX-2 is a target of either GSK-3β or β-catenin in MC3T3-E1 osteoblasts, we inhibited GSK-3β activity with LiCl or activated β-catenin and measured COX-2 protein levels by immunoblot. Inhibition of GSK-3β activity with LiCl led to a significant increase in COX-2 protein levels after 5 hours (Fig. 6A). No increase in COX-2 was seen after only 1 hour of treatment. Next, β-catenin was activated in osteoblasts by expressing a constitutively active β-catenin molecule. Cells were transfected with a c-myc epitope tagged, β-catenin molecule comprising the last 4 of 5 potential serine and threonine GSK-3β phosphorylation sites in the *N*-terminus converted to alanine (termed m-β-catenin). m-β-Catenin cannot be phosphorylated by GSK-3β and, therefore, accumulates and translocates into the nucleus. In Figure 6B, immunofluorescence analysis of m-β-Catenin-expressing cells using an antibody to the c-myc tag shows that the mutant goes both to sites of cell–cell attachment and to the nucleus (Fig. 6B). Multiple groups of pooled, stably transfected colonies from several independent transfections were screened by immunoblot analysis for expression of m-β-catenin and for Cox-2 protein levels. Cells expressing m-β-catenin consistently expressed significantly higher levels of COX-2 protein than control, vector alone–transfected MC3T3-E1 cells (Fig. 6B). These results indicate that increasing the stabilized signaling pool of β-catenin is sufficient for increasing COX-2 protein levels in osteoblasts.

Discussion

In this study, we sought to determine whether the signaling functions of GSK-3β and β-catenin are modulated in osteoblasts subjected to FSS. The data presented here make the novel demonstration that both GSK-3β activity and β-catenin signaling are altered by laminar FSS. These results suggest an important role for β-catenin and GSK-3β signaling, not only in the anabolic response of osteoblasts to FSS, but also in the general response of cells to mechanical stimulation. The process of mechanotransduction, the conversion of a mechanical stimulus into a biochemical response, is known to occur in osteoblasts in response to FSS: however, the molecular mechanisms remain largely unknown. Our studies suggest that both GSK-3β and β-catenin signaling are mechano-responsive and may mediate the transmission of mechanical stimuli. GSK-3β is an unusal kinase in that it is normally catalytically active. Phosphorylation of serine 9 on GSK-3β results in its inactivation [49–51], which happens in response to FSS as we demonstrate in this study. There are conflicting reports on the effects of GSK-3β serine 9 phosphorylation on β-catenin signaling. Some studies show a direct effect of this phosphorylation event on β-catenin signaling and others show no effect [52–54]. In fact, GSK3-β is a widely expressed kinase that regulates multiple cellular functions including glycogen synthesis, cytoskeletal organization, and apoptosis. Thus, GSK-3β

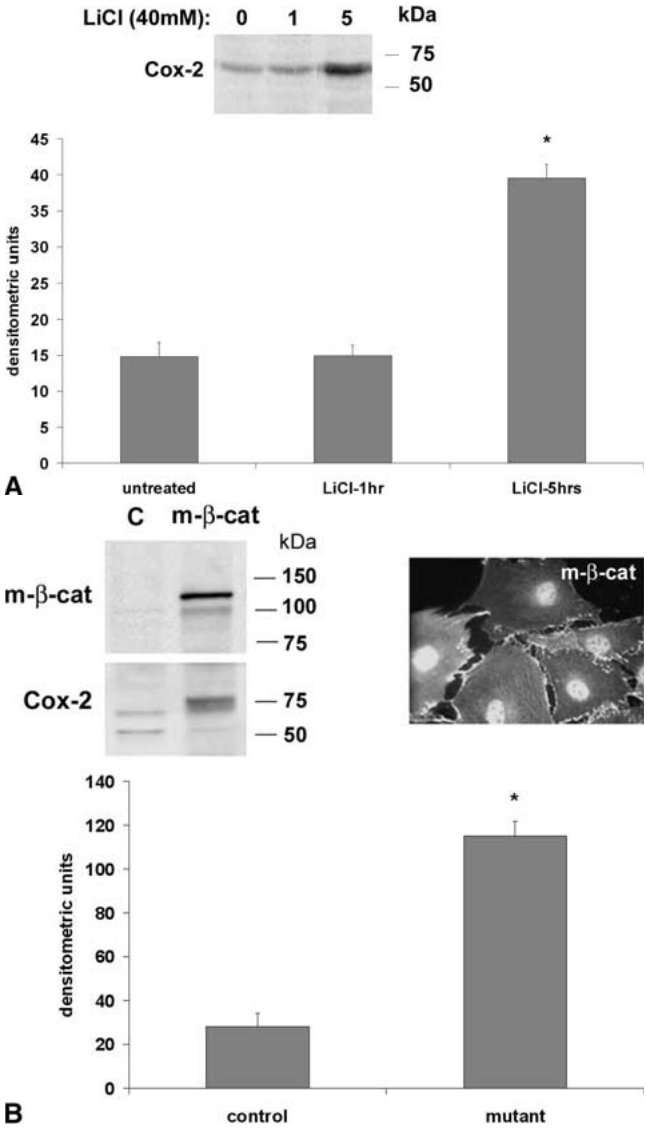

Fig. 6. Expression of a constitutively active β-catenin molecule or inhibition of GSK-3β activity increases COX-2 levels. (**A**) MC3T3-E1 cells were treated for 1 or 5 hours with 40 mM LiCl to inhibit GSK-3β activity, and the levels of Cox-2 protein were determined by immunoblot analysis. Equal protein from whole cell lysates was loaded onto SDS-PAGE gels and transferred to nitrocellulose. Inhibition of GSK-3β with LiCl for 5 hours significantly increased COX-2 levels in osteoblasts. Control versus 5-hours, $P < .05$, Student t test. (**B**) Stable cell lines expressing a constitutively active β-catenin, m-β-catenin, or vector alone control cells (C) were created. Immunofluorescence analysis using an antibody to the c-myc epitope tag revealed that m-β-catenin localized at cell–cell interfaces and accumulated in the nucleus as expected. Whole cell lysates of multiple groups of pooled, drug-resistant colonies from independent transfections were loaded onto SDS-PAGE gels, transferred to nitrocellulose, and screened by immunoblot for expression of the m-β-catenin by using an antibody directed against the c-myc epitope tag and for expression of COX-2. Lysate from MC3T3-E1 cells expressing the constitutively active β-catenin, m-β-catenin, is shown on the right and control, pcDNA3 transfected MC3T3-E1 lysate (C) is shown on the left, m-β-Catenin–expressing cells expressed significantly higher levels of COX-2 protein than did control MC3T3-E1 cells. Control vs. m-β-catenin, $P < .05$, Student t test.

is likely affecting β-catenin signaling as well as non-β-catenin-mediated signal transduction pathways. Our studies also show that Akt is phosphorylated in response to FSS, with a time-course similar to that of GSK-3β, thereby making Akt a likely candidate for modulating the FSS-induced inactivation of GSK-3β. However, the activity of GSK-3β is controlled by many upstream signals including, but not limited to Akt, integrin-linked kinase (ILK), protein kinase A (PKA), and Wnts (reviewed in [55]). In fact, we cannot rule out that FSS induces the secretion of Wnt proteins that act in an autocrine fashion to regulate phosphorylated GSK-3β. This is an intriguing idea given the recent studies that show that mutations in a Wnt receptor, LRP 5, cause high or low bone mass phenotypes depending on the site of mutation [56, 57]. Investigation into these and other mechanisms for the FSS regulation of GSK-3β and β-catenin will be the goal of future studies.

In addition to GSK-3β modulation, a second potential mechanism for regulation of β-catenin signaling during FSS is proposed in this study. FSS significantly decreased the levels of *N*-cadherin associated with β-catenin. Cadherins, which interact directly with β-catenin, are known to modulate the signaling activity of β-catenin by sequestering β-catenin to the plasma membrane and preventing its translocation [58]. It is of interest to note that only the association of β-catenin with N-cadherin, but not with cadherin-11 was decreased by FSS. Further ,N-cadherin is known to have potential signaling properties with respect to cell differentiation, including differentiation of cells of the osteoblast lineage [59, 60]. Modulation of the β-catenin/N-cadherin complex by FSS may be a general mechanism for regulating fluid β-catenin signaling in osteoblasts.

In this study, we also identified COX-2 as a potential target gene for GSK-3β and β-catenin in osteoblasts. Of interest, a recent study demonstrated that in renal medullary interstitial cells COX-2 levels were increased by inactivation of GSK-3β, but not β-catenin activation [61]. However, consistent with our studies, Kim et al. [35] demonstrated in chondrocytes that expression of a mutant, active β-catenin molecule significantly increased COX-2 levels. Furthermore, TCF-like promoter elements have been reported to exist in the COX-2 promoter [24, 32, 33, 48], and increased levels of nuclear β-catenin/TCF correlate with increased levels of COX-2 in various cell types [24, 32–34]. In this study, we have demonstrated that osteoblast β-catenin and TCF proteins interact directly with one TCF-binding element in the COX-2 promoter. This is probably the strongest evidence in support of a direct modulation of COX-2 expression by β-catenin/TCF in osteoblasts. It may be that such regulation occurs only in the right molecular environment. For example, in 2003, Araki et al. [48] demonstrated that β-catenin regulation of COX-2 expression in hepatocellular carcinoma cells required co-

expression of K-ras. It is intriguing that COX-2 in both chondrocytes and osteoblasts is responsive to activated β-catenin, suggesting that cells of a common progenitor may be specifically tuned to respond to activation of β-catenin. Whether β-catenin also contributes to the FSS induction of COX-2 is still unclear. However, we predict based on our results that β-catenin contributes to the FSS induction of COX-2, but that it is probably not the sole regulator of COX-2 expression in response to FSS. It is likely that FSS-induced molecular signaling requires a complement of transcription factors to fully mediate the response to mechanical signals. In fact, the COX-2 promoter is known to contain other potential regulatory elements including CRE, NF-kB, Sp1, and AP2 sites [40]. In summary, we propose that FSS modulates the activity of Akt, GSK-3β, and β-catenin and that these molecules are important signaling components of mechanotransduction in osteoblasts. Furthermore, β-catenin and GSK3-β regulate the expression of COX-2, which may be one of many targets of these proteins in osteoblasts.

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