European Review for Medical and Pharmacological Sciences 2014; 18: 3149-3155

Activation of β-catenin stimulated by mechanical strain and estrogen requires estrogen receptor in mesenchymal stem cells (MSCs)

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Abstract. – BACKGROUND AND OBJECTIVES: Mechanical stimulation and hormones act via interconnected signaling pathways to influence the function of bone cells. Estrogen receptor (ER) and β-catenin play important role in bone formation and have implicated in mechanotransduction in bone cells. To investigate the interaction between mechanotransduction and estrogenic signaling in mesenchymal stem cells (MSCs), this study examined the effect of mechanical strain and estrogen on activation of β-catenin in MSCs, and the role of ER in response to mechanical strain and estrogen in MSCs.

MATERIALS AND METHODS: MSCs were exposed to mechanical strain (2%, 1 Hz) and estrogen (100 nM). The ER inhibitor, ICI182,780 was used to assess the role of ER in activation of β-catenin stimulated by mechanical strain and estrogen. Changes of activated β-catenin in the nuclei were determined by immunoflourescent test. The expression of β-catenin was detected by western blotting.

RESULTS: Mechanical strain and estrogen augment, respectively, activation of β-catenin and accumulation of activated β-catenin in the nuclei of MSCs. Combined treatment with estrogen and mechanical strain had higher levels of activated β-catenin than the cells exposed to mechanical strain or estrogen. After MSCs were pre-treated by ICI182,780, the level of activated β-catenin expression induced by mechanical stain or estrogen was depressed. Meanwhile, ICI182,780 also blocked the effect of combined stimulation on activation of β-catenin in MSCs.

CONCLUSIONS: Our study demonstrates that mechanical strain and estrogen both promote the levels of activated β-catenin in MSCs. Estrogen receptor implicates in activation of β-catenin stimulation by mechanical strain and estrogen in MSCs.

Key Words:
Mechanical strain, Estrogen, Mesenchymal stem cells (MSCs), Estrogen receptor, β-catenin.

Introduction

Bone marrow derived mesenchymal stem cells (MSCs) is vital to bone remodeling and recovery from disease. Osteogenic differentiation of MSCs contributes to renewal of bone[1]. Mechanical stimulation and estrogen have been regard as two important microenvironmental factors for regulation of MSCs differentiation, which promote osteoblastogenesis of MSCs[2,3].

The responsiveness of bone to mechanical stimulation in skeletal development and disease is associated with estrogen level[4-6]. In MSCs, estrogen receptor (ER) is found to be involved in response mechanical stimulation and estrogen[2,7]. The effect of estrogen on bone cells is mediated by ER, especially ERα. ER binds to estrogen response elements (EREs) and other transcription factors, resulting in the transcription of target genes in response to estrogen. The effect of estrogen also is mediated by membrane and cytoplasmic ER without the involvement of nuclear ER[8]. Estrogen has been shown to influence estrogen receptor expression, and enhanced osteogenic activity via ER activity in MSCs[2,9]. In osteoporotic MSCs, content of ER is significantly less than that of normal cells[10]. Knocking out ER expression in mice has blocked the osteogenic response to mechanical stimulation[11,12]. A hypothesis may be of interest in investigating mechanism of bone cell's response to mechanical stimulation, which is that effect of estrogen on number and activity of ER is associated with effectiveness of bone cells' response to strain[6,13].

Many signaling pathways have been proved to link with other pathways in mechanotransduction[14]. β-catenin is a key component of classical

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Wnt signaling pathway. Wnt signaling can inhibit degradation of β-catenin, and contributes to interaction of activated β-catenin with transcription factors to regulate downstream genes[15]. Knocking out the β-catenin in mesenchymal progenitor cells has completely inhibited differentiation towards osteoblasts, maintained MSCs distribution into chondrocytes[16]. In osteoblasts, the interaction of ER with β-catenin takes part in response to mechanical strain[17]. The potential interactions of signaling pathways may play important role in skeletal development and disease. However, less is known about the interaction between mechanotransduction and estrogenic signaling in MSCs.

We examined the effect of mechanical strain and estrogen on activation of β-catenin in presence and absence of special ER inhibitor. This study investigated the hypothesis that activation of β-catenin stimulated by mechanical strain and estrogen involves estrogen receptor in MSCs.

Materials and Methods

Reagents

17β-Estradiol and type I collagen were purchased from Sigma (Saint Louis, MO, USA). The ICI182,780 was purchased from Tocris (Bristol, UK). Anti-active-β-catenin was purchased from Millipore Corporation (Billerica, MA, USA). Total β-catenin and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell Culture

MSCs were isolated as described previously[18]. Briefly, the bone marrow was obtained from femur and tibia of 3-month-old Sprague-Dawley rats and was suspended in DMEM-LG containing 15% charcoal stripped foetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified 5% CO_2 incubator. Non-adherent cells were removed by changing culture medium. The adherent cells were passaged upon reaching 80% confluence with 0.25% Trypsin/EDTA solution (Hyclone, Logan, VT, USA) at room temperature, subcultured into T25 culture flasks. Immunophenotyping of MSCs were analyzed by flow cytometry to assure that the cells were positive for CD90, CD44, CD29, but negative for CD31, CD45, CD11b. Then, cells (P3-P4) were incubated in DMEM-LG containing 2% FBS for 24h before experiments described below.

Application of Mechanical Stretch and Culture Conditions

The culture chambers with flexible silicon membranes had been pre-coated with type I collagen overnight. MSCs were seeded onto culture chambers at a density of 4×10^5/well, and were subjected to a biaxial cyclic strain with 2% surface elongation at 1 Hz. Cells were maintained in similar culture chambers without mechanical strain served as the controls. In some experiments, cells were exposed to 17β-estradiol (100 nM), and ER inhibitor, ICI182,780 (100 nM).

Immunofluorescence

After treatment, the cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were permeabilized with 0.5% Triton X-100 in PBS. The cells were incubated in 1% bovine serum albumin (BSA) in PBS + Tween (PBST) to block unspecific binding of the antibodies. Then the cells were incubated with primary antibody (active-β-catenin) overnight at 4°C in humidified chamber. Fluoroscein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was used for 1h in the dark. The cells were washed with PBS (4×10 min) and visualized by confocal microscopy.

Western Blotting

The cells were lysed with a buffer (150 mM NaCl, 50 mM Tris HCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS and complete protease inhibitor tablets) on ice. Protein were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with freshly prepared TBS/T containing 5% non-fat dry milk, and incubated with primary antibodies overnight at 4°C. Then, these membranes were washed with TBS/T, and incubated with secondary antibody conjugated with horseradish peroxidase at room temperature. After washing with TBS/T, the proteins were detected using enhanced chemoluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA).

Statistical Analysis

All experiments were performed at a minimum of three times. Resultant data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA. Differences were considered statistically significant for p values smaller than 0.05.

Results

MSCs were exposed to estrogen for 0 min, 15 min, 30 min, 1 h and 6 h. As shown in Figure 1A, a significant increase of activated β-catenin in nuclei was observed in response to estrogen for 1h by confocal microscopy. As compared with control cells, Western blots of whole of cells lysates showed an increase in activated β-catenin protein after estrogen for 1 h to 6 h (Figure 1B). There was a lack of significant difference in estrogen-related activated β-catenin from 1 h to 6 h.

MSCs were subjected to mechanical strain for 0 min, 15 min, 30 min, 1 h and 6 h. Accumulation of activated β-catenin in the nuclei of MSCs

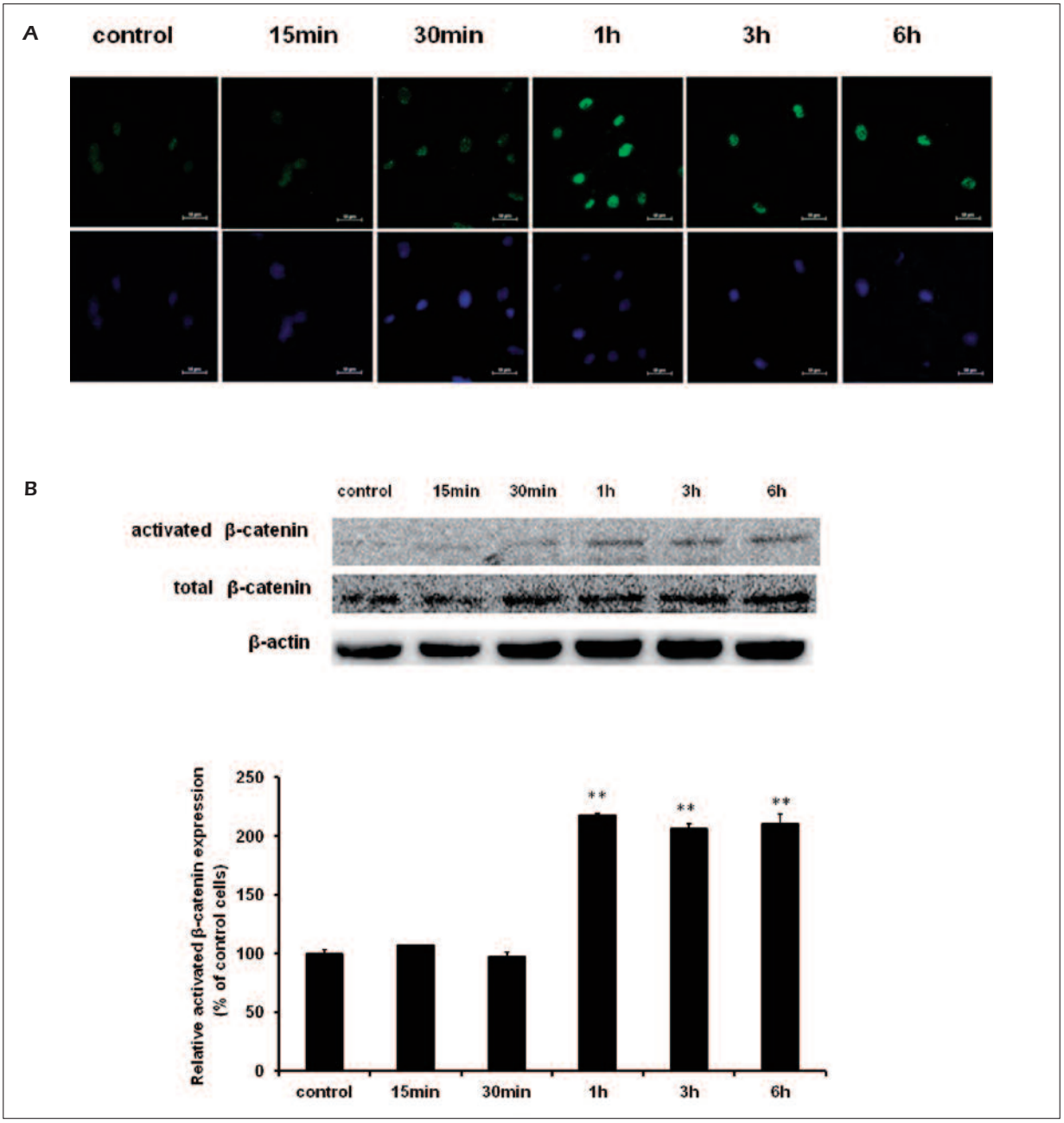

Figure 1. Effect of Estrogen on β-catenin in MSCs. ***A,*** MSCs were subjected to estrogen for 0min to 6 h. Activated β-catenin (green staining) and DAPI staining were determined by confocal microscopy. ***B,*** MSCs were exposed to estrogen for 0 min to 6 h. Total and activated β-catenin were examined by western blot. The relative levels of activated β-catenin were normalized by the levels of β-actin (**$p < 0.01$ vs control).

began to increase after mechanical stimulation for 1 h. The results of western blots showed that activated β-catenin in whole cells significantly increased in response to mechanical strain for 1 h to 6 h as compared with control cells (Figure 2A). But, a significant change of activated β-catenin in cells was not observed after mechanical strain for 1 h to 6 h (Figure 2B). To investigate effect of combined treatment with estrogen and mechanical strain, MSCs were treated with estrogen treatment for 1 h before mechanical strain for 1 h. The effect of combined stimulation approximated to the arithmetic sum of the increase caused by respective factors, and had higher levels of activated β-catenin than the cells exposed to estrogen or mechanical strain.

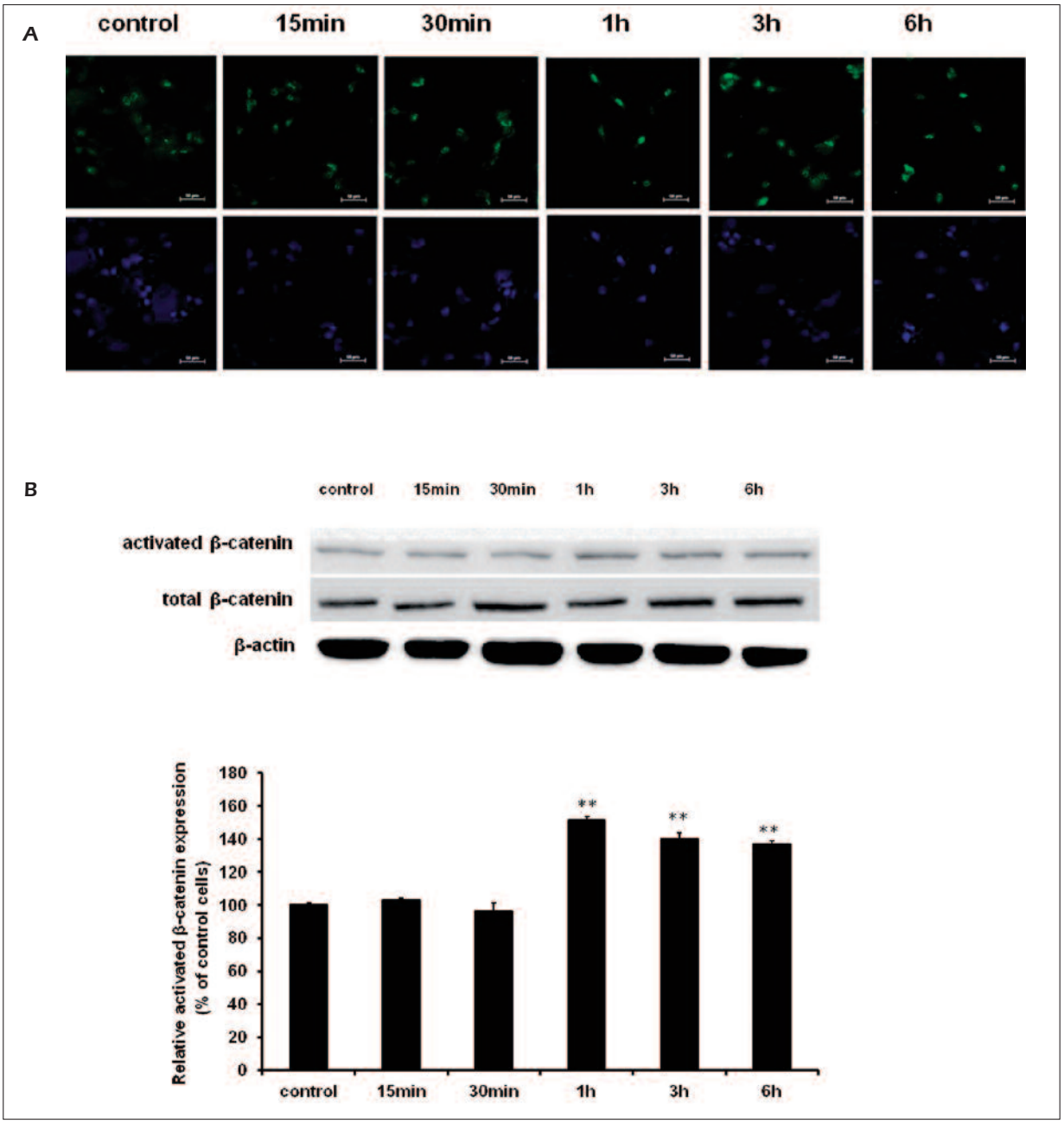

Figure 2. Effect of mechanical strain on β-catenin in MSCs. ***A,*** MSCs were subjected to mechanical strain for 0 min to 6 h. Activated β-catenin (green staining) and DAPI staining were determined by confocal microscopy. ***B,*** MSCs were strained 0 min to 6 h. Total and activated β-catenin were examined by western blot. The relative levels of activated β-catenin were normalized by the levels of β-actin (**$p < 0.01$ vs control).

The ER inhibitor ICI182,780 (100 nM) was used to evaluate whether activation of β-catenin stimulated by estrogen or mechanical strain was mediated by ER. Pre-treating MSCs with ICI182,780 for 1 h before estrogen resulted in an significant decrease of activated β-catenin, as compared with the cells exposed to estrogen. MSCs pre-treated with ICI182,780 for 1 h with the subsequent exposure to mechanical strain had lower levels of activated β-catenin than the cells exposed to mechanical strain. The addition of ICI182,780 had a significant effect on activated β-catenin protein in MSCs exposed to combined stimulation (Figure 3).

Discussion

Although, some studies showed that estrogen influences β-catenin in neurons and colon cancer[19,20], less is known about the effect of estrogen on activation of β-catenin in MSCs. In order to identify duration of stimulation, MSCs were exposed to estrogen in different time. The data presented here showed that estrogen stimulated accumulation of activated β-catenin in the nuclei, and increased level of activated β-catenin in MSCs. Activated β-catenin can translocate to the nucleus, and trigger downstream signals to regulate osteogenic potential of MSCs[15].

Cross-talk between estrogen and Wnt/β-catenin signaling pathways through interaction between ER and β-catenin has been shown in study on colon cancer cells[20]. Membrane and cytoplasmic ER contribute to estrogenic signaling, besides nuclear ER. Estrogen has been shown to regulate the stability of β-catenin by triggering the association of ER with membrane or cytoplasmic signaling pathways[19]. Studies on differentiation and proliferation of human osteoblasts

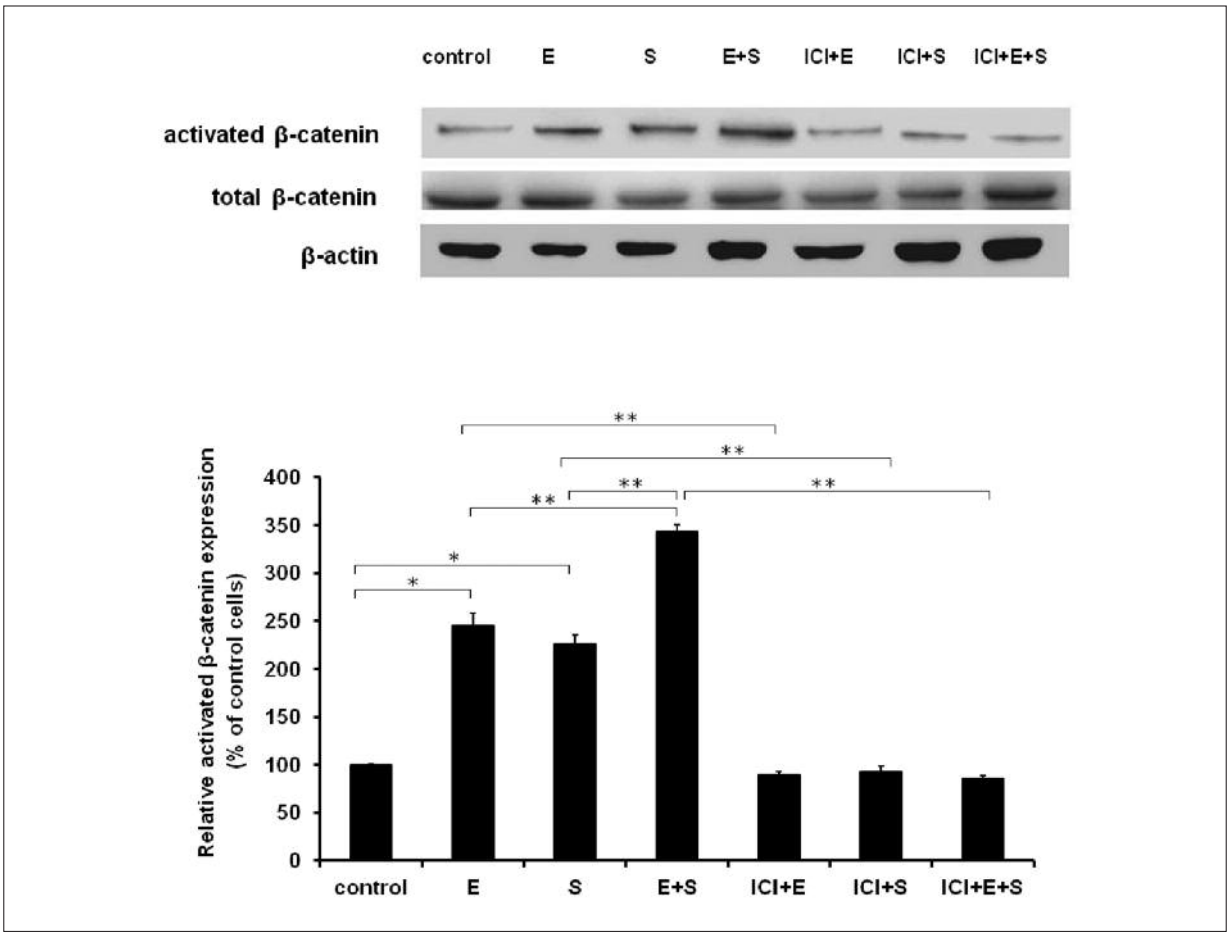

Figure 3. Effect of estrogen and mechanical strain on β-catenin in presence and absence of ER inhibitor in MSCs. The relative levels of activated β-catenin were normalized by the levels of β-actin (E vs control, S vs control, $*p < 0.05$; E+S vs S, E vs E+ICI, S vs S+ICI, E+Svs E, E+S vs E+S+ICI, $**p < 0.01$).

in vitro estrogen-deficiency model reported functional interaction between ER and β-catenin[21]. When ER activity is absent or declined, activity of β-catenin and ER/β-catenin interactions are destroyed in osteoblasts[17,21]. Here, MSCs were subjected to ER inhibitor in order to mimic estrogen deficiency *in vitro*. Although the precise mechanisms of estrogen activated β-catenin in MSCs remain unclear, our data demonstrated that ICI182,780 blocked estrogen-induced activation of β-catenin, suggesting that effect of estrogen on activation of β-catenin involves ER in MSCs.

Buer Sen et al[22,23] demonstrated that mechanical strain inhibits adipogenisis of MSCs via activated β-catenin, suggesting that β-catenin plays a role in MSCs response to mechanical strain. In osteoblasts, Armstrong et al[17] found that mechanical strain activates β-catenin translocates to nucleus. In agreement with previous studies, our results indicated that mechanical strain promotes activation of β-catenin in MSCs, as compared with unstained cells. In this study, the ER inhibitor was used to examine the role of ER in strain-induced active β-catenin. Although the interaction of Wnt with LRP5/6 may be partially involved in mechanotransduction in osteoblasts, Sunters et al[24] found that mechanical strain initiates the interaction of ER with PI3K/AKT-dependent activation of β-catenin, and strain-induced active β-catenin is independent of Wnt/LRP. ER plays an important component in the strain-dependent activation of β-catenin in osteoblasts[17]. We also found that ER involves in strain-related activation of β-catenin in MSCs.

Here, pre-treating MSCs with estrogen before mechanical strain had higher levels of activated β-catenin expression than the cells exposed to estrogen or mechanical strain, but the synergistic effect of estrogen and mechanical strain on β-catenin activation was not seen in MSCs. Estrogen has been reported to result in translocation of ER from cytoplasm to the nucleus[6]. It may be speculated that translocation of ER may explain that the effect of combined treatment-induced activation of β-catenin in our experiments. The cascade mechanisms of interaction between ER and β-catenin in response to estrogen and mechanical strain in MSCs remain to be fully defined. Our *in vitro* studies showed that the ER inhibitor completely blocked the effect of combined stimulation on activated β-catenin to return to base line, further suggesting that activation of β-catenin in MSCs stimulated by two factors (mechanical strain and estrogen) involve ER.

Conclusions

Our findings demonstrated that mechanical strain and estrogen both induce activation of β-catenin in MSCs. Activation of β-catenin stimulation by mechanical strain and estrogen receptor involve ER in MSCs. Further investigation of interaction between mechanotransduction and estrogenic signaling will be provided new insights into bone cell's response to mechanical stimulation in bone physiology and disease.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (No. 30770534, 11272225, 10972149).

Conflict of Interest

The Authors declare that: haven't financial and personal relationships with other people or organizations that could inappropriately influence this work

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