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Estrogen receptor α- (ERα), but not ERβ-signaling, is crucially involved in mechanostimulation of bone fracture healing by whole-body vibration

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ABSTRACT

Mechanostimulation by low-magnitude high frequency vibration (LMHFV) has been shown to provoke anabolic effects on the intact skeleton in both mice and humans. However, experimental studies revealed that, during bone fracture healing, the effect of whole-body vibration is profoundly influenced by the estrogen status. LMHFV significantly improved fracture healing in ovariectomized (OVX) mice being estrogen deficient, whereas bone regeneration was significantly reduced in non-OVX, estrogen-competent mice. Furthermore, estrogen receptors α (ERα) and β (ERβ) were differentially expressed in the fracture callus after whole-body vibration, depending on the estrogen status. Based on these data, we hypothesized that ERs may mediate vibration-induced effects on fracture healing. To prove this hypothesis, we investigated the effects of LMHFV on bone healing in mice lacking ERα or ERβ. To study the influence of the ER ligand estrogen, both non-OVX and OVX mice were used.

All mice received a femur osteotomy stabilized by an external fixator. Half of the mice were sham-operated or subjected to OVX 4 weeks before osteotomy. Half of each group received LMHFV with 0.3 g and 45 Hz for 20 min per day, 5 days per week. After 21 days, fracture healing was evaluated by biomechanical testing, μCT analysis, histomorphometry and immunohistochemistry.

Absence of ERα or ERβ did not affect fracture healing in sham-treated mice. Wildtype (WT) and ERβ-knockout mice similarly displayed impaired bone regeneration after OVX, whereas ERα-knockout mice did not. Confirming previous data, in WT mice, LMHFV negatively affected bone repair in non-OVX mice, whereas OVX-induced compromised healing was significantly improved by vibration. In contrast, vibrated ERα-knockout mice did not display significant differences in fracture healing compared to non-vibrated animals, both in non-OVX and OVX mice. Fracture healing in ERβ-knockout mice was similarly affected by LMHFV as in WT mice. These results suggest that ERα-signaling may be crucial for vibration-induced effects on fracture healing, whereas ERβ-signaling may play a minor role.

1. Introduction

Bone fractures require suitable mechanical stimulation at the site of injury for an optimal healing process [1–3]. It has been shown that external mechanical stimulation, including whole-body low-magnitude high-frequency vibration (LMHFV), provoked anabolic effects on the skeleton in preclinical [4–7] and clinical studies in healthy and osteoporotic individuals [8, 9]. Although there are also studies showing no effect of LMHFV on the intact skeleton [10–12], whole-body vibration was supposed to provide a non-invasive, cost-effective and safe treatment to improve compromised fracture healing in osteoporotic patients. However, preclinical data about the influence of LMHFV on bone repair are heterogeneous and seem to depend on the estrogen status of the animals. It was shown that LMHFV provoked beneficial effects on fracture healing in estrogen-deficient rodents [13–17], whereas several studies demonstrated no or even a negative effect on bone regeneration in estrogen-competent animals [13, 15–19]. This indicates a major role for estrogen in mechanostimulation of fracture healing and implies that LMHFV might only be effective in confined target populations. However, the underlying mechanisms remain poorly understood.

On a molecular level, it has been shown that in non-ovariectomized (non-OVX) mice, LMHFV upregulated the expression of estrogen receptor β (ERβ) in the fracture callus [16], which is regarded to inhibit bone formation in response to mechanical loading [20, 21]. In contrast, in OVX mice, ERα expression was significantly increased in the callus by vibration therapy [16, 22]. ERα is considered the most essential receptor for mediating estrogen effects on bone [23–25]. Furthermore signaling

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through ERα was shown to be crucial for anabolic effects of mechanical loading [20, 26–28]. Further studies suggested that the ratio between ERα and ERβ might be important. ERβ was demonstrated to reverse or inhibit ERα-mediated gene transcription, indicating a "Ying/Yang" relationship between the two receptors [29, 30]. In general, ERs can activate transcription by binding directly to the genome at estrogen responsive elements (EREs) or indirectly through interaction with transcription factors [31, 32]. Furthermore, both ERs can exert signaling both in the presence and absence of their ligand estrogen [33]. The activated genes are highly dependent on the receptor subtype and whether or not estrogen is present [34]. This underlines the hypothesis that both ERs may play different roles in gene regulation of mechanotransduction, depending on the estrogen status.

To address this hypothesis, we investigated the roles of ERα and ERβ in vibration-induced effects on fracture healing using ER-knockout mice. To study the influence of the ER ligand estrogen, we used both non-OVX and OVX mice. Absence of ERα completely abolished the effects induced by OVX and LMHFV, whereas the lack of ERβ had no influence. These results suggest a critical role of ERα-, but not of ERβ-signaling in the effects of mechanostimulation of bone fracture healing. Therefore we conclude that ERα-signaling is crucial for vibration-induced effects on fracture healing, whereas ERβ-signaling may play a minor role.

2. Material and methods

2.1. Animal care and surgical procedure

All experiments were performed according to German Guidelines of Animal Research on the Protection of Animals as well as the ARRIVE guidelines and were approved by the local ethical committee (No. 1248, Regierungspräsidium Tübingen, Germany). Female C57BL/6J wildtype (WT) mice were provided by the University Medical Center Ulm, while female homozygous B6.129P2-Esr1$^{tm1Ksk/J}$ (ERα-KO) and B6.129P2-Esr2$^{tm1Unc/J}$ (ERβ-KO) mice were provided by Charles River Laboratories (Wilmington, USA). At the age of 12 weeks, half of the mice were subjected to bilateral OVX as described previously [35] to induce estrogen-deficiency, whereas the remainder was sham operated (non-OVX). Four weeks later, all mice underwent standardized unilateral femur osteotomy as described previously [16, 35]. Briefly, the osteotomy was created at the right femur diaphysis using a 0.4 mm gigli wire saw (RISystem, Davos, Switzerland) and stabilized by a semi-rigid external fixator (RISystem). Half of all mice received LMHFV (Table 1). All mice were sacrificed 21 days after surgery using an isoflurane overdose.

2.2. LMHFV

Mice were placed on custom-made vibration platforms for 20 min per day for 5 days per week, starting with the third postoperative day, as described previously [16]. Vibration settings were 0.3 g sinusoidal peak-to-peak acceleration and 45 Hz frequency. The amplitude and frequency were continuously recorded using integrated accelerometers at the platform (Sensor KS95B.100, measurement amplifier Innobeamer L2, Software Vibromatrix; IDS Innomic GmbH, Salzwedel, Germany).

Table 1
Overview about experimental groups and number of used mice per group.

genotype	WT				ERα-KO				ERβ-KO			
sham-OVX	6	8			6	8			8	6		
OVX			7	8			6	7			9	7
sham-vibration	6		7		6		6		8		9	
vibration		8		8		8		7		6		7

Furthermore, vibration transduction to the bone was verified *in vivo* in one test mouse directly at the external fixator. Therefore, an inertial measurement unit (MPU-9250, Invensense, San Jose, USA) was mounted on the external fixator, measuring acceleration and angular rate in all three dimensions. The MPU-9250 breakout board was connected to an Arduino board and data were recorded at a sample rate of 500 Hz (Supplemental Fig. S1 A). Data analysis was performed using MS Excel, confirming an undamped transduction of amplitude and frequency (Supplemental Fig. S1 B). The control mice were sham-vibrated on the same platforms without activation of the vibration generator.

2.3. Biomechanical testing and μCT analysis

Fractured femurs were explanted after euthanasia and subjected to a non-destructive three-point bending test as described previously [16]. Briefly, a force of maximal 4 N was applied on top of the cranio-lateral callus side and deflection was measured using a materials testing machine (Zwick, Ulm, Germany). Flexural rigidity was calculated from the slope of the linear region of the force-deflection curve. After biomechanical testing, bones were fixed in 4% paraformaldehyde and scanned in a μCT device (Skyscan 1172, Kontich, Belgium) at a resolution of 8 μm using a voltage of 50 kV and 200 μA. To determine the apparent bone mineral density (BMD), two phantoms with a defined density of hydroxyapatite (250 mg/cm^3 and 750 mg/cm^3) were included within each scan. To determine the total volume (TV) and bone volume to total volume fraction (BV/TV), a global threshold of 641.9 mg hydroxyapatite/cm^3 [36] was used to distinguish between mineralized and non-mineralized tissue. μCT analysis was done with the 3D analysis software from Skyscan (NRecon, DataViewer, CTAn). The volume of interest comprised the entire callus between the fractured cortices.

2.4. Histomorphometry and immunohistochemistry

Following μCT analysis, bone specimens were subjected to histology as described previously [37]. Longitudinal sections of 7 μm were stained with Giemsa or Safranin O for histomorphometric tissue quantification. The amounts of bone, cartilage and fibrous tissue in the callus between the two inner pinholes were determined using image analysis software (Leica DMI6000 B; Software MMAF Version 1.4.0 MetaMorph®; Leica, Heerbrugg, Switzerland). The number and surface of osteoblasts (NOb/BPm, ObS/BS) were determined using Toluidine blue staining. Osteoblast are defined as toluidine blue-positive, cubic-shaped cells with visible cytoplasm, located directly on the bone surface. The flat-shaped bone lining cells on the bone surface were excluded from the analysis. The number and surface of osteoclasts (NOc/BPm, OcS/BS) were determined using tartrate-resistant alkaline phosphatase (TRAP) staining. Osteoclasts were defined as TRAP-positive cells with two or more nuclei, directly located on the bone surface with a visible resorption lacunae between the bone matrix and the cell. Bone cells and surface were evaluated using Osteomeasure system (Osteometrics, Decatur, USA). Paraffin-embedded sections were used for immunohistochemical stainings. ERα and ERβ expression were detected using a previously published protocol [16]. Briefly, primary antibody against ERα (1:75, sc-542, Santa Cruz Biotechnology, Dallas, USA) and ERβ (1:40, sc-8974, Santa Cruz Biotechnology), and secondary antibodies against rabbit IgG (goat anti-rabbit IgG-biotin, Invitrogen, Darmstadt, Germany) were used for immunohistochemistry. Since the used antibody against ERβ was recently published to be non-specific in human samples [38], we included sections from the respective KO mice as negative controls to confirm specific staining.

In general, serial sections from the middle of the fracture callus were used to stain Giemsa, Safranin O, Toluidine blue, TRAP, ERα, ERβ and the respective IgG controls. One section per mouse was used per each staining.