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The skeletal responsiveness to mechanical loading is enhanced in mice with a null mutation in estrogen receptor-β

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Saxon LK, Robling AG, Castillo AB, Mohan S, Turner CH. The skeletal responsiveness to mechanical loading is enhanced in mice with a null mutation in estrogen receptor-β. *Am J Physiol Endocrinol Metab* 293: E484–E491, 2007. First published May 29, 2007; doi:10.1152/ajpendo.00189.2007.—Mechanical loading caused by physical activity can stimulate bone formation and strengthen the skeleton. Estrogen receptors (ERs) play some role in the signaling cascade that is initiated in bone cells after a mechanical load is applied. We hypothesized that one of the ERs, ER-β, influences the responsiveness of bone to mechanical loads. To test our hypothesis, 16-wk-old male and female mice with null mutations in ER-β (ER-$\beta^{-/-}$) had their right forelimbs subjected to short daily loading bouts. The loading technique used has been shown to increase bone formation in the ulna. Each loading bout consisted of 60 compressive loads within 30 s applied daily for 3 consecutive days. Bone formation was measured by first giving standard fluorochrome bone labels 1 and 6 days after loading and using quantitative histomorphometry to assess bone sections from the midshaft of the ulna. The left nonloaded ulna served as an internal control for the effects of loading. Mechanical loading increased bone formation rate at the periosteal bone surface of the mid-ulna in both ER-$\beta^{-/-}$ and wild-type (WT) mice. The ulnar responsiveness to loading was similar in male ER-$\beta^{-/-}$ vs. WT mice, but for female mice bone formation was stimulated more effectively in ER-$\beta^{-/-}$ mice ($P < 0.001$). We conclude that estrogen signaling through ER-β suppresses the mechanical loading response on the periosteal surface of long bones.

bone formation rate; osteoporosis

BONE SHAPE AND ARCHITECTURE are strongly influenced by their mechanical loading environment. Weight-bearing exercise can strengthen bones by stimulating new bone formation, and this is greatest in regions with large tissue deformation (mechanical strain) (30). Thus, large mechanical strains will initiate bone formation, whereas the lack of sufficient mechanical strains will result in local bone resorption and bone loss. This phenomenon is observed in people exposed to extreme loading conditions such as elite gymnasts at one extreme and astronauts at the other (8, 37).

The responsiveness of the skeleton to mechanical loading varies during different stages of life, and this appears to be associated with changes in the body's endocrine environment. The skeleton is clearly more responsive during the pre- and peripubertal years when growth hormone is the main stimulant of bone formation, compared with the postpubertal years in girls when estrogen levels rise (3, 11, 23, 39). After puberty, the skeletal responsiveness to mechanical loading has been shown to diminish, particularly on the outer periosteal surface where estrogen is known to inhibit bone formation (1). These findings, along with others (4, 5, 12), suggest that mechanical loading and estrogen share a common mechanistic pathway.

The estrogen receptors (ERs) are found in osteoclasts and osteoblasts and can be stimulated by both estrogen and mechanical loading (2, 9, 41). Estrogen binds to the ER located in the cytoplasm causing dimerization of the ER, after which it translocates to the nucleus, where it mediates gene transcription via classical estrogen response element (ERE) or via AP-1 and Sp1 sites that interact with fos/jun complexes; in turn, this may lead to an increase in cell number and activity. The ER, more specifically ER-α, is also important for initiating a response to mechanical strain, for when it is absent the cortical bone's response to loading is diminished 70% (19). There are, however, two ERs: ER-α and ER-β. The role of the second ER, ER-β, in loading is unknown.

ER-β was identified 10 yr after the first ER, ER-α, was cloned in 1986 (15). These receptors share a high homology in the DNA-binding domain (>90%) but not as high in the ligand-binding domain (55%) (15, 16). This suggests that ER-β would recognize and bind to similar EREs as ER-α but that each receptor has a distinct group of ligands. On the basis of the phenotype of transgenic mice lacking these receptors, ER-α appears to enhance bone formation on the endocortical (inner cortical surface) and trabecular bone surfaces in males and females, whereas ER-β inhibits periosteal (outer cortical surface) bone formation and has no effect on trabecular bone formation (21, 25, 34, 36, 38). Thus, ER-β and ER-α appear to have opposing effects on periosteal bone formation, and this is evident in gene transcription. Microarray analysis shows that, in bone, gene expression stimulated by estrogen is 85% higher in ER-$\beta^{-/-}$ mice compared with wild-type (WT) mice, suggesting that ER-β reduces ER-α-regulated gene transcription in bone (22).

Given the evidence supporting opposing effects of ERs in bone, we proposed that ER-β inhibits bone formation in response to mechanical loading. This proposal is consistent with findings in cell culture: ER-β-deficient primary osteoblasts show markedly enhanced proliferation in response to mechanical strain compared with WT cells (20). We sought to confirm these findings in vivo using a mouse model with a null mutation in the ER-β gene. We examined the role of ER-β in skeletal mechanotransduction for male and female mice.

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METHODS

Animal model. Male and female heterozygous (ER-$\beta^{+/-}$) mice were mated, and the offspring containing ER-$\beta^{+/+}$ (WT) and ER-$\beta^{-/-}$ alleles were used in the experiments. In this mouse model, ER-β was deleted by the homologous recombination with *neo* insertion into exon 3 of the ER-β gene (7). At 3 wk of age, genotyping of tail-biopsy DNA for ER-β was accomplished by PCR using the primers P1 (5′-TAT CCC TAG CTC TGG AAG GC-3′), P2 (5′-AAG CGC ATG CTC CAG ACT GC-3μ), and P3 (5′-ACA TTT ATA TCA GAT CAT CTC TGC-3′), which yield a 381-bp fragment for WT mice and a 237-bp fragment for the ER-$\beta^{-/-}$ allele. The mice were housed two per cage and provided standard mouse chow and water ad libitum throughout the study. When the mice reached 16 wk of age, they were subjected to short bouts of mechanical loading at the right ulna, as described below. At 18 wk of age the mice were euthanized, and bone samples were dissected and stored in 70% ethanol at −20°C. All procedures performed in this experiment were in accordance with the Indiana University Institutional Animal Care and Use Committee guidelines.

Serum IGF measurement. Immediately after the mice were euthanized, blood was collected via cardiac puncture. Blood was stored in a no-additive vial on ice and spun at 3,000 *g* for 10 min. Serum was transferred to a 0.5-ml microcentrifuge tube and stored at −80°C until processing. Serum samples were assayed for IGF using a radioimmunoassay (RIA). Briefly, IGFs in serum are bound to IGF-binding proteins, and it is known that IGF-binding proteins produce artifacts in IGF RIA. Therefore, IGFs were separated from IGF-binding proteins using the Bio-Spin separation protocol with BioGel P-10 columns in 1 M acetic acid. This rapid acid gel filtration protocol has been validated previously (24). The IGF concentration was determined by RIA, using recombinant human IGF-I as a tracer and standard and rabbit polyclonal antiserum as described earlier (24). The cross-reactivity of IGF-II in IGF-I RIA is <0.5%. The sensitivity of the IGF-I RIA is <50 ng/l. The intra- and interassay coefficients of variation (CVs) for IGF-I assay are <10% (24).

Dual X-ray absorptiometry. At 16 wk of age the animals were weighed, and a whole body dual X-ray absorptiometry scan was completed using a PIXImus II small animal densitometer (Lunar, GE-Healthcare). Measured variables include percent fat mass, percent lean tissue mass, bone mineral content (BMC) of the whole body, lumbar spine (L3-5), and right hind leg. The densitometer also reported bone mineral density (BMD), which is BMC divided by the projected skeletal area. However, bone area was found to vary considerably because the mice could not be positioned exactly the same. Therefore, BMC is the only outcome measure reported. Short-term reliability for this procedure in four animals scanned four times with interim repositioning showed an average CV of 2.9% for BMC.

Peripheral quantitative computed tomography. Bone density was measured in several bones using a high-resolution peripheral quantitative computed tomography (pQCT) machine. Dissected bones were cleaned of musculature and placed in 70% ethanol for pQCT measurements. pQCT was used to measure BMC, bone area, and volumetric BMD at the cortical bone site of the midfemur and at the predominantly trabecular bone sites of the lumbar vertebrae and distal femur. Each bone was positioned in a plastic tube containing 70% ethanol and centered in the gantry of the pQCT machine (Norland Medical Systems Stratec XCT Research SA+). For the lumbar spine, height of the vertebral body was measured and a midslice taken at 50% of the height of L5. The femur was scanned at the midslice, taken at 50% of the total bone length, and three slices were taken at 15, 17.5, and 20% of the total length measured from the distal end of the femur, and the mean values from these slices were recorded. Analysis was performed using Stratec software with the following parameters for cortical bone (femur midshaft): *threshold 1*: 710, *threshold 2*: 710, peel mode: 2, contour mode: 1; and for trabecular bone (L5 and distal femur): *threshold 1*: 169, *threshold 2*: 710, peel mode: 2, contour mode: 3. The CV for measurements of BMC, area, and volumetric BMD (vBMD) was ~3.7%.

Biomechanical testing. The effect of ER-β deletion on mechanical properties of the femur was determined from a random sample of WT and ER-$\beta^{-/-}$ mice. Femurs were positioned cranial side up across the lower supports of the Vitrodyne V1000 materials testing machine (Liveco, Burlington, VT). The supports had a span width of 9.0 mm, and the bones were fixed in place with a ~0.1-N static preload before being loaded to failure in three-point bending using a crosshead speed of 0.2 mm/s. During testing, force vs. displacement measurements were collected, from which we determined ultimate force (in N) and stiffness (in N/mm).

Microcomputed tomography. Midshaft of the left ulnae were scanned ex vivo using microcomputed tomography (μCT) (uCT20; Scanco Medical, Bassersdorf, Switzerland) to measure bone length, bone diameter, and minimum and maximum second moments of area (I_{MIN} and I_{MAX}, respectively). Scans were conducted using a slice thickness of 9 μm. The second moment of area estimates the bone's resistance to bending by considering the distribution of the bone in the cross section. The plane of greatest bone rigidity is known as the major axis, and the second moment of area along this plane is known as I_{MAX}. The plane of least bending rigidity is known as the minor axis, and the second moment of area along that axis is denoted I_{MIN}. In the ulna, the greatest osteogenic response occurs on the medial and lateral bone surfaces, and the largest structural change after loading is seen in I_{MIN}. For this reason, I_{MIN} of the left ulna midshaft was used to calculate how much mechanical strain was applied to the right ulna for each mouse.

Mechanical strain measurement during dynamic axial loading. The peak mechanical strain at the lateral aspect of the ulnar midshaft was measured for male and female WT and ER-$\beta^{-/-}$ mice. The ulnae were brought to room temperature in a saline bath (over 2 h), and soft tissue was dissected to reveal the lateral surface of the midshaft ulna. A single element strain gauge (EA-06-015DJ-120; Vishay Measurement Group) was bonded to the ulna using M-bond adhesive (Vishay Measurement Group). The forearm was loaded in cyclic axial compression, using the same device and frequency used for in vivo loading (Fig. 1). Voltage output at each load magnitude of 1.0, 1.2, 1.4, 1.6, and 1.8 N was measured with an oscilloscope. Voltage was converted to strain, as previously described (29). The moment of area at the corresponding site where the strain gauge was attached was measured using μCT with 9-μm slice thickness. The images were imported into image-processing software (Scion Image) to calculate I_{MIN} and the maximum section diameter in the I_{MIN} plane. From the midsection μCT measurements and mechanical strain data, strain was estimated for the periosteal surface for each animal loaded in vivo, as previously described (29).

Ulnar loading protocol. Eight male and eight female WT and ER-$\beta^{-/-}$ mice were randomly assigned to either a high, medium, or low mechanical loading group. While under isofluorane anesthetic, the right forearm from each group was axially loaded for 60 cycles/day using a 2 Hz haversine waveform daily for 3 consecutive days. When a downward force is applied to the ulna the bone bends in the medial-lateral direction, resulting in compression of the medial surface and tension on the lateral surface. The greatest bending moment occurs near the midshaft. The left forearms were not loaded and were used as a nonloaded control to allow side-to-side comparisons for loading effects. All mice were allowed normal cage activity in between loading bouts. All mice received intraperitoneal injections of calcein (30 mg/kg body wt; Sigma Chemical, St. Louis, MO) 2 days after the last day of loading and alizarin (50 mg/kg body wt; Sigma Chemical) 7 days after the last day of loading.

Histomorphometry. The right and left ulnas were processed for histomorphometry. Prior to processing, the midsection of the ulnae were marked using a gray lead pencil. The ulnas were then dehydrated in graded alcohol (70–100%) and cleared in xylene, each for 3 h, and infiltrated with methyl methacrylate (MMA) for 24 h using a vacuum

Fig. 1. Application of load to the mouse forelimb. A downward force was applied to the upright ulna, causing the bone to bow laterally, resulting in compression of the medial surface and tension on the lateral surface.

tissue processor (Shandon Pathcentre Tissue Processor). The ulnas were then stored in MMA (and 3% softener) inside a vacuum oven for 4 days. The ulnas were embedded in MMA and kept in a water bath set at 24°C for 2 days, then at 60°C overnight or until the plastic was set. Using a diamond wire saw (Histo-saw; Delaware Diamond Knives), transverse sections (~50 μm) were removed from the ulna diaphysis at the midsection and mounted unstained on standard microscope slides. The sections were then ground down, using sandpaper to thin sections (~20 μm) to improve clarity.

One slice per limb was read at ×200 on a fluorescence microscope (Nikon Optiphot; Nikon). Using the Bioquant digitizing system (R&M Biometrics, Nashville, TN), the following data were derived from the periosteal and endocortical surface under ×160 magnification: single-label perimeter, no label perimeter, double-label perimeter, and double-label area. From these primary data, the following were calculated for each surface: mineralizing surface [MS/bone surface (BS), %], mineral apposition rate (MAR; μm/day), and lamellar bone formation rate (BFR/BS; $\mu m^3 \cdot um^2 \cdot yr^{-1}$). For surfaces with genuine single labels but no double label, a value of 0.03 was used for MAR to enable BFR to be calculated.

Statistical analysis. A two-way analysis of variance was used to determine the main effects of genotype and sex on phenotypic measurements. When the interaction term was significant ($P < 0.05$), post hoc comparisons were conducted using a Fisher's protected least significant difference test. Differences between right (loaded) and left (nonloaded) ulna were tested for significance using paired *t*-tests. Simple regression models were used to determine the relationship between applied mechanical strain and bone formation measurements. Strain-adjusted bone formation measures were computed using regression residuals.

RESULTS

Mouse characteristics. Whereas there were no significant effects of the ER-$\beta^{-/-}$ genotype on body weight, body composition was significantly affected in female mice. The fat mass of female ER-$\beta^{-/-}$ mice was significantly greater than WT mice (Table 1). In contrast, male ER-$\beta^{-/-}$ mice showed no difference in any measure of body composition compared with WT mice. The ulnae of ER-$\beta^{-/-}$ mice were slightly longer (~2%) than WT mice (Table 3). Others (21) have observed higher serum insulin-like growth factor levels in female ER-$\beta^{-/-}$ mice. We found that serum IGF-I levels were no different ($P > 0.3$) between WT and ER-$\beta^{-/-}$ mice in both males (195.5 ± 40.2 and 180.2 ± 33.4 ng/ml, respectively; n = 12–16) and females (168.0 ± 30.1 and 184.1 ± 39.4 ng/ml, respectively; n = 11–12).

Bone densitometry. To determine the impact of ER-β on skeletal mass, BMC was measured for the whole skeleton in vivo in ER-$\beta^{-/-}$ and WT mice. In addition, regional analysis was performed to determine lumbar spine and hind leg BMC. Absence of ER-β had no significant effect on BMC at any site in either male or female mice compared with WT (Table 1).

pQCT was used to measure BMC, bone area and vBMD of sites rich in cortical (midfemur) or trabecular bone (lumbar vertebra or distal femur) (Table 2). The absence of ER-β had no effect on BMC, bone area, or BMD of the midfemur in either male or female mice. However, ER-$\beta^{-/-}$ mice had significantly lower vBMD at the lumbar spine and distal femur.

Table 1. *Body composition and bone mass of the WT and ER-$\beta^{-/-}$ mice at 16 wk of age*

	Female		Male		2-Way ANOVA Results		
	WT	ER-$\beta^{-/-}$	WT	ER-$\beta^{-/-}$	Genotype	Sex	Interaction
	n = 12	n = 7	n = 13	n = 13			
Body weight, g	19.9±0.8	21.0±1.2	25.4±3.5	25.6±3.8	NS	$P < 0.0001$	NS
Lean mass, g	16.1±0.7	16.9±0.8	20.8±2.9	21.4±3.5	NS	$P < 0.0001$	NS
Fat mass, g	2.3±0.2	3.0±0.5*	3.2±0.8	3.2±0.5	$P = 0.02$	$P = 0.001$	$P = 0.04$
Total body BMC, mg	335±27	333±7	361±43	352±69	NS	$P = 0.12$	NS
L3-5 BMC, mg	19±2	18±2	18±2	17±4	NS	$P = 0.09$	NS
Hind leg BMC, mg	35±3	35±1	43±7	41±9	NS	$P = 0.001$	NS

Values are means ± SD ($P > 0.15$). WT, wild type; ER-β, estrogen receptor-β; NS, not significant; BMC, bone mineral content. *Significantly different from WT ($P < 0.05$).

Table 2. *Bone mass, bone area, and bone density determined using pQCT for WT and ERβ$^{-/-}$ mice at 16 wk of age*

	Female		Male		2-Way ANOVA Results		
	WT	ER-β$^{-/-}$	WT	ER-β$^{-/-}$	Genotype	Sex	Interaction
Midfemur							
Total BMC, mg/mm	1.120±0.114	1.141±0.134	1.312±0.191	1.249±0.174	NS	$P < 0.0001$	NS
Total area, mm^2	1.533±0.180	1.544±0.245	1.856±0.307	1.769±0.255	NS	$P < 0.0001$	NS
Total vBMD, mg/cm^3	733.2±50.7	729.8±53.7	709.0±27.8	707.3±44.1	NS	$P = 0.03$	NS
Distal femur							
Total BMC, mg/mm	1.431±0.213	1.385±0.205	1.410±0.234	1.382±0.232	NS	NS	NS
Total area, mm^2	2.699±0.345	2.834±0.387	2.980±0.425	2.962±0.299	NS	$P = 0.01$	NS
Total vBMD, mg/cm^3	532.8±44.6	493.3±54.1	476.0±39.1	466.0±43.2	$P = 0.01$	$P < 0.0001$	NS
L4 vertebra							
Total BMC, mg/mm	2.025±0.329	1.964±0.311	1.948±0.294	1.733±0.175	$P = 0.04$	$P = 0.02$	NS
Total area, mm^2	5.104±0.311	5.221±0.479	5.256±0.374	5.121±0.329	NS	NS	NS
Total vBMD, mg/cm^3	395.8±55.4	375.2±37.8	370.2±46.8	338.0±22.2	$P = 0.01$	$P = 0.002$	NS

Values are means ± SD ($P > 0.15$, $n = 15$–22). pQCT, peripheral quantitative computed tomography; vBMD, volumetric bone mineral density.

These results suggest that sites rich in trabecular bone are preferentially affected by the null mutation in ER-β.

Biomechanics. Biomechanical properties of the femur were not significantly affected in ER-β$^{-/-}$ mice (Table 3). In female mice, the stiffness was greater in ER-β$^{-/-}$ compared with WT, but this difference did not achieve statistical significance.

μCT. μCT measurements indicate no difference in bone geometry of the ulna midshaft between ER-β$^{-/-}$ and WT mice (Table 4). However, the estimated strain applied to the ulna during mechanical loading (based on strain gauge data) was ~27% lower in the female ER-β$^{-/-}$ mice compared with WT mice ($P < 0.05$), demonstrating that ulnae from female ER-β$^{-/-}$ sustain less mechanical strain per unit load, suggesting that they are stiffer than WT ulnae.

Response of the ulna to mechanical loading. After 3 days of high-magnitude loading, there was a significant increase in periosteal bone formation (loaded vs. nonloaded ulnae) in male and female ER-β$^{-/-}$ and WT mice ($P < 0.05$). Bone formation on the endocortical surfaces of the ulnae was not significantly affected by mechanical loading. Therefore, our discussion will focus on the responses observed on the periosteal surface. As noted above, the mechanical strains varied with genotype. To account for this variation, bone formation parameters were adjusted to the residuals of regressions with mechanical strain (Table 5). Male mice with the ER-β$^{-/-}$ genotype responded the same to mechanical loading as the age-matched WT mice. However, mechanical loading increased bone formation 3.6-fold more in female ER-β$^{-/-}$ mice subjected to the highest load compared with WT (Fig. 2). This genotypic difference in response was due to both increased MAR and increased MS (MS/BS), each of which contributes to the BFR (Fig. 3). Interestingly, all bone formation measurements were higher in nonloaded control ulnae of female ER-β$^{-/-}$ mice, suggesting an effect of ER-β on periosteal bone formation even without applying mechanical loading.

DISCUSSION

Transgenic mice provide a unique opportunity to assess the role a particular receptor plays in mechanotransduction. However, in loading experiments it is important to take into consideration the effect knocking out this receptor has on the bone phenotype. ER-β$^{-/-}$ mice showed only small effects of the gene mutation on the skeletal phenotype. The ulnae from female ER-β$^{-/-}$ mice were observed to engender less mechanical strain under a given load, meaning they were stiffer (femora from female ER-β$^{-/-}$ mice were also stiffer than WT, but this difference did not achieve statistical significance). If the same magnitude of load is applied to the ulna of the knockout and WT mouse, the stiffer bone will experience less strain compared with a smaller, more compliant bone. To account for this, we estimated how much strain each animal received on the basis of strain gauge measurements and ulna midshaft bone geometry and then calculated the increase in bone formation per unit increase in mechanical strain. Subsequently, we found that the increase in periosteal bone formation per unit increase in strain was about threefold greater in the female ER-β$^{-/-}$ mice compared with WT mice. Thus, equal changes in strain did not result in equal changes in bone formation in the ER-β$^{-/-}$ mice. In contrast, there was no difference in the increase in periosteal bone formation per unit increase in strain in male ER-β$^{-/-}$ and WT mice. Thus, we propose that ligand-bound ER-β reduces the response to mechanical loading on the periosteal surface of cortical bone.

The increased responsiveness to mechanical loading observed in female ER-β$^{-/-}$ mice supports measurements made

Table 3. *Biomechanical parameter measured using 3-point bending of femora from male and female WT and ER-β$^{-/-}$ mice at 16 wk of age*

	Female		Male		2-Way ANOVA Results		
	WT	ER-β$^{-/-}$	WT	ER-β$^{-/-}$	Genotype	Sex	Interaction
Ultimate force, N	14.9±0.5	14.7±0.8	18.6±1.0	17.0±0.8	NS	$P < 0.0001$	NS
Stiffness, N/mm	73.8±2.6	83.5±4.0	83.3±3.9	83.1±5.0	NS	NS	NS

Values are means ± SD ($P > 0.15$, $n = 10$–17).

Table 4. *Geometry of the ulna measured using μCT for mice at 16 wk of age*

	Female		Male		2-Way ANOVA Results		
	WT	ER-$\beta^{-/-}$	WT	ER-$\beta^{-/-}$	Genotype	Sex	Interaction
Bone length, mm	13.41±0.57	13.73±0.44	14.18±0.57	14.38±0.40	$P = 0.005$	$P < 0.0001$	NS
I_{MIN}, mm^4	0.0035±0.0009	0.0036±0.0010	0.0045±0.0012	0.0040±0.0010	NS	$P = 0.001$	$P = 0.12$
I_{MAX}, mm^4	0.0146±0.0036	0.0149±0.0034	0.0205±0.0057	0.0184±0.0043	NS	$P < 0.0001$	$P = 0.13$
Microstrain/Newton, με/N	931±243	676±141*	716±135	765±207	$P = 0.002$	$P = 0.06$	$P < 0.0001$

Values are means ± SD ($P > 0.15$, $n = 26$–42). μCT, microcomputed tomography; I_{MIN}, minimum second moments of area; I_{MAX}, maximum second moments of area. *Significantly different from WT ($P < 0.05$).

by Lee et al. (20) using cultured bone cells. They found that 10 min of mechanical strain resulted in a 125 ± 40% increase in the number of osteoblast-like cells derived from female ER-$\beta^{-/-}$ mice but that cells from WT mice increased by only 61 ± 25%. Interestingly, the first ER discovered, ER-α, appears to have an opposite effect on the bone's response to loading. Cortical bone from mice lacking ER-α showed a significantly reduced mechanoresponsiveness compared with WT mice (19). These results support the hypothesis that signaling through ER-α enhances bone's mechanoresponsiveness, whereas ER-β signaling suppresses it (31). Considering the enhanced mechanoresponsiveness of the ulna in adult female ER-$\beta^{-/-}$ mice, we might expect to observe differences in cortical bone mass and morphology in these mice due to hyperresponsiveness to loading during skeletal growth and development. However, we could not find any difference in cortical bone mass or size (except ulnar length) when comparing ER-$\beta^{-/-}$ mice to WT mice. This suggests that either ER-β exerts limited effect on mechanoresponsiveness during growth or the amount of load required to engage the ER-β signaling pathway is supraphysiological and not normally achieved during routine daily activities.

It has been shown that animals with null mutations for ER-α and ER-β have distinct skeletal abnormalities (34), and distinct signaling pathways can be activated by ER-α and ER-β (10, 13, 27, 33). In addition, both ER-α and ER-β can activate classical EREs in response to estrogen but signal in opposite ways at an AP-1 site in the presence of estradiol; through ER-α 17β-estradiol activates transcription, whereas through ER-β 17β-estradiol inhibits transcription (27). Thus, determining the role of each receptor in bone mechanotransduction has been a challenge. Interpretation of results from ER-null mice is difficult due to multiple effects of the null mutation on several tissues. For instance, ER-$\alpha^{-/-}$ mice have elevated serum estrogen levels, and some have observed elevated serum IGF-I levels in ER-$\beta^{-/-}$ mice (although we could not confirm this in our colony). Although we acknowledge the shortcomings of the mouse models, like the one used in this study, we note that the importance of ERs in the skeletal response to mechanical loading has been supported by cell culture experiments and more recently in humans. In culture, mechanical strain enhances ER-α signaling in osteoblast-like cells, and when additional ER-α is added proliferation is enhanced in response to loading (12, 19, 40). Conversely, when ERs are blocked by selective ER modulators such as tamoxifen and ICI 182,780, the proliferative osteoblastic response to mechanical loading is eliminated (4, 5). Thus, ERs appear to mediate the proliferative response of osteoblasts to mechanical loading in cell culture; ER-α seems to promote this response, whereas ER-β appears to suppress it (20).

Others (28, 35) have observed that specific genetic polymorphisms at the ER-α locus (PvuII polymorphic site) appear to modulate the mechanosensitivity of bone. A cross-sectional study found only active girls with the Pp genotype had a higher bone density and cortical thickness compared with less active girls, and no differences in bone were detected between girls with the PP and pp genotypes (35). The results of a 4-yr exercise intervention (28) showed that men in the exercise group with PP or Pp genotypes showed an increase in lumbar spine bone density, whereas no changes were detected in men with the pp genotype. Although these results need to be confirmed in other populations, they suggest that people with

Table 5. *Responsiveness to high-magnitude mechanical loading of the ulna in WT and ER-$\beta^{-/-}$ mice at 16 wk of age*

	Female		Male		2-Way ANOVA Results		
	WT	ER-$\beta^{-/-}$	WT	ER-$\beta^{-/-}$	Genotype	Sex	Interaction
	$n = 10$	$n = 10$	$n = 8$	$n = 9$			
L-BFR, $\mu m^3 \cdot \mu m^2 \cdot yr^{-1}$	41.5±30.9	144.4±68.2*	91.0±64.2	85.4±74.5	$P = 0.02$	NS	$P = 0.01$
NL-BFR, $\mu m^3 \cdot \mu m^2 \cdot yr^{-1}$	1.4±1.1	9.0±9.1	8.1±13.1	6.0±5.9	NS	NS	$P = 0.08$
rBFR, $\mu m^3 \cdot \mu m^2 \cdot yr^{-1}$	40.1±30.9	135.5±68.8*	82.8±64.6	79.5±72.0	$P = 0.03$	NS	$P = 0.02$
L-MAR, μm/day	0.09±0.34	0.55±0.24*	0.39±0.22	0.34±0.25	$P = 0.03$	NS	$P = 0.007$
NL-MAR, μm/day	0.03±0.00	0.07±0.05	0.07±0.08	0.06±0.05	NS	NS	NS
rMAR, μm/day	0.06±0.34	0.48±0.25*	0.32±0.24	0.28±0.23	$P = 0.04$	NS	$P = 0.01$
L-MS/BS, %	46.4±15.4	89.9±19.2*	68.6±30.7	60.0±15.3	$P = 0.007$	NS	$P = 0.001$
NL-MS/BS, %	12.7±10.2	29.7±13.2*	21.7±12.6	23.1±8.6	$P = 0.02$	NS	$P = 0.04$
rMS/BS, %	33.7±16.0	60.2±22.6*	46.9±28.9	36.9±21.9	NS	NS	$P = 0.02$

Values are means ± SD ($P > 0.15$). L and NL, loaded and nonloaded ulnae, respectively; BFR, bone formation rate; rBFR, relative bone formation rate; MAR, mineral apposition rate; rMAR, relative mineral apposition rate; rMS/BS, relative mineralizing surface/bone surface. Relative bone formation parameters, i.e., loaded − nonloaded, show increases caused by mechanical loading. Data are presented for the high-load group. The measurements were adjusted to account for differences in peak mechanical strain. *Significantly different from WT ($P < 0.05$).

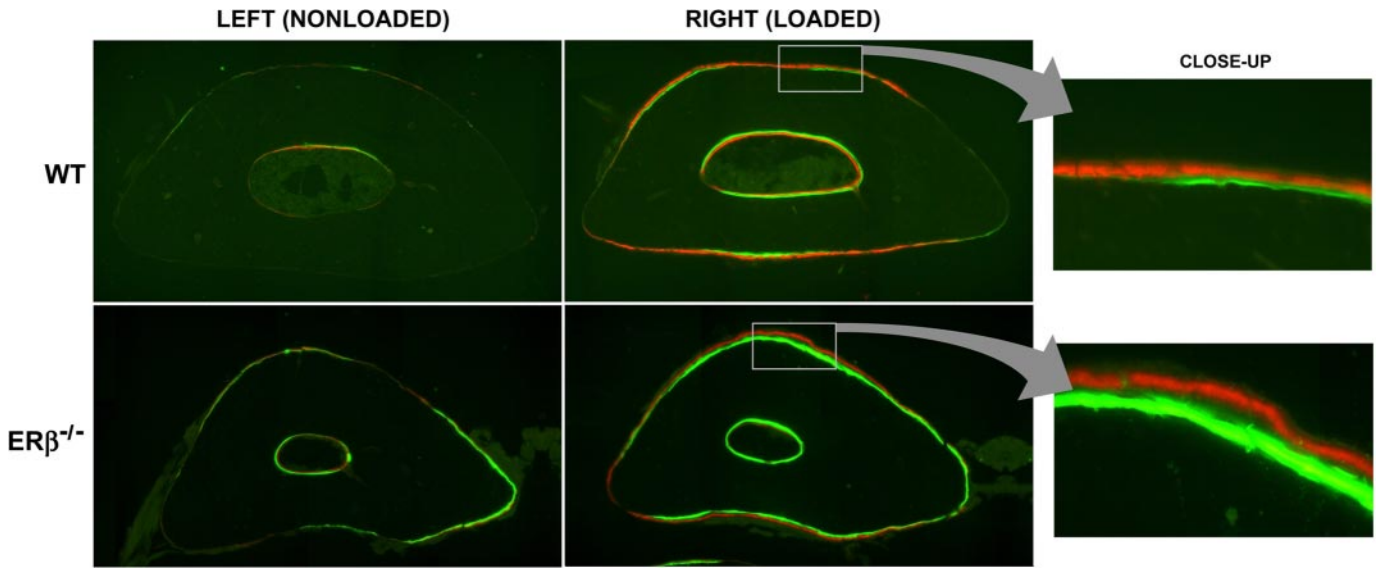

Fig. 2. Midshaft ulnar tissue sections from nonloaded (control) and loaded forearms among female wild-type (WT) and estrogen receptor (ER)-$\beta^{-/-}$ mice given fluorochrome injections after loading show a robust bone formation response on the medial (*insets*) and lateral surfaces of the loaded ulna. This response was ~3-fold greater in ER-$\beta^{-/-}$ mice compared with WT.

specific polymorphisms of the ER-α gene are more sensitive to exercise than others. The influence of ER-β on mechanotransduction in humans has not been studied; however, associations have been found between ER-β genotype and bone density in pre- and postmenopausal Asian women (18, 26).

The influence of ER-β deficiency had some effects on the skeletal phenotypes of our mice. ER-$\beta^{-/-}$ mice had slightly longer ulnae compared with WT mice. Lindberg et al. (21) found increased serum IGF-I levels and femur length in ER-$\beta^{-/-}$ mice compared with WT mice and a strong correlation between the two, suggesting a causal relationship between serum IGF-I and femur length. We did not find elevated serum IGF-I in our ER-$\beta^{-/-}$ mice, and the increase in bone length we observed (~2%) was less than that reported by Lindberg et al. (6%) (21). Furthermore, we observed increased bone length in both male and female ER-$\beta^{-/-}$ mice, so this apparent effect of

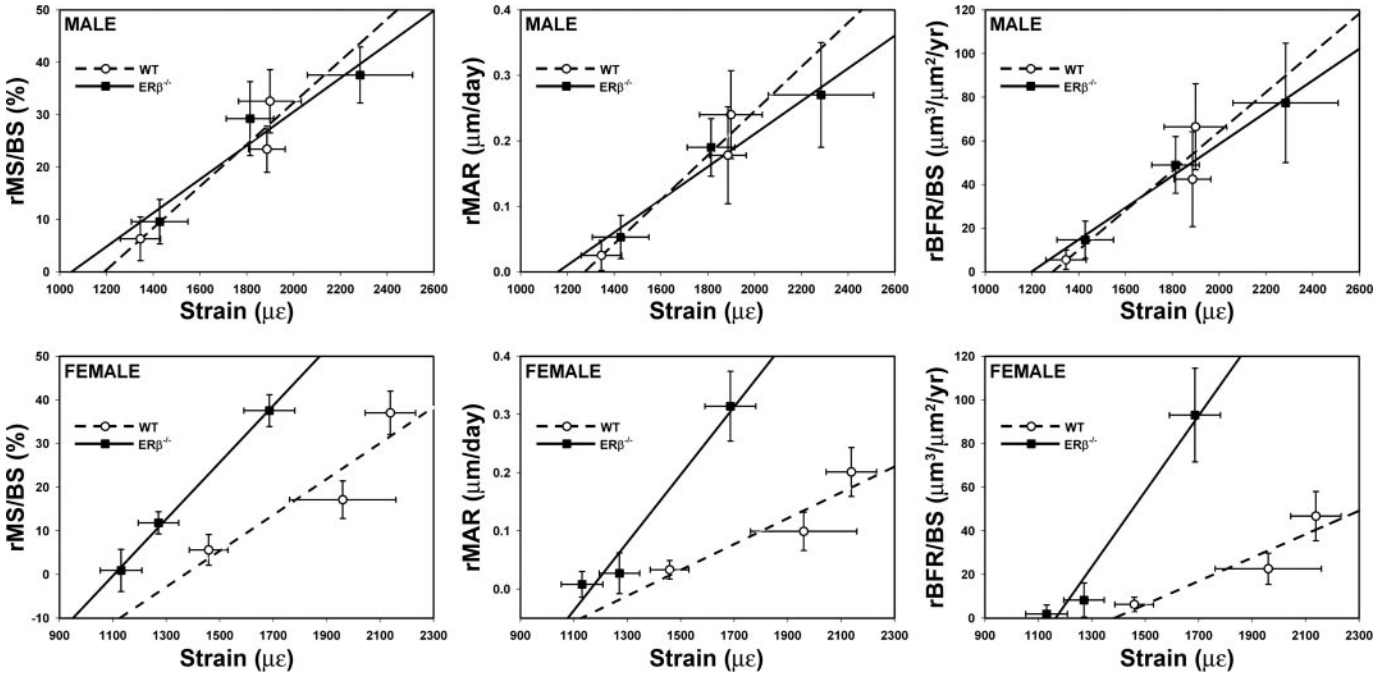

Fig. 3. The relationships between mechanical strain applied to the ulna midshaft and bone formation parameters were unaffected by genotype in male ER-$\beta^{-/-}$ mice, but in female mice the null mutation of ER-β increased the responsiveness of the bone to mechanical strain. Relative mineralizing surface (rMS/BS) and mineral apposition rate (rMAR) each contribute to the relative bone formation rate (rBFR). Both rMS/BS and rMAR were higher in female ER-$\beta^{-/-}$ mice compared with WT. As a result, the slope of the curve relating rBFR to mechanical strain was 3.6-fold higher in ER-$\beta^{-/-}$ mice compared with WT. Bone formation parameters are presented as relative (loaded − nonloaded) values.

ER-$\beta^{-/-}$ on bone growth does not correlate with the sex-specific enhancement of mechanical responsiveness observed only in female ER-$\beta^{-/-}$ mice.

The lack of observed differences in serum IGF-I levels between WT and ER-$\beta^{-/-}$ female mice in this study suggests that the positive effect of ER-$\beta^{-/-}$ on the periosteal response to mechanical loading cannot be explained on the basis of ER-β modulation of endocrine IGF-I action. However, we cannot rule out the possibility that alterations in local IGF-I actions could in part contribute to enhanced anabolic response to loading in the ER-$\beta^{-/-}$ female mice. In this regard, lack of ER-β may lead to increased local expression of IGF-I in response to loading, or regulate IGF-I action at the local level by modulating production of IGF-binding proteins. In addition, estrogen could modulate IGF-I action by interacting with the IGF-I receptor downstream of receptor activation (6). Because of the established role of IGF-I receptor signaling in mediating the response to mechanical loading in osteoblasts in vitro (14), further studies are needed to evaluate whether sensitivity to locally produced IGF-I is altered in response to loading in ER-$\beta^{-/-}$ female mice.

In our mice, the development of fat mass in females appeared to be influenced by ER-$\beta^{-/-}$, which differs from the finding of Lindberg et al. (21) that ER-β had no effect on fat mass. We cannot say whether the increased fat content is associated with increased serum estrogen levels, but earlier work (21, 34) has not found any difference in estrogen levels in female ER-$\beta^{-/-}$ compared with WT mice. The cortical bone phenotype in ER-$\beta^{-/-}$ mice was similar to WT, but the trabecular bone density was reduced in ER-$\beta^{-/-}$ mice. A similar bone phenotype in ER-$\beta^{-/-}$ mice was described by Sims et al. (34) and Lindberg et al. (21). For the most part, our ER-$\beta^{-/-}$ mice had similar skeletal characteristics as those previously reported for this mouse strain.

In summary, others (17) have proposed that ER-α signaling is involved in osteoblast mechanotransduction. Our data clearly show that ER-β also plays a role in bone mechanotransduction and suggest that ER-β functions as a negative modulator of the periosteal response to mechanical loading. This phenomenon fits with the findings that estrogen limits the loading response on the periosteal surface after puberty (1, 32). Our data suggest that suppression of ER-β signaling might augment the positive effects of exercise, potentially increasing bone size and reducing the risk of fracture later in life. Perhaps selective ER modulators will be most effective for improving bone strength if they simultaneously suppress ER-β activity in bone while enhancing ER-α activity.

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