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Estrogen Receptors $\alpha$ and $\beta$ Have Different Gender-Dependent Effects on the Adaptive Responses to Load Bearing in Cancellous and Cortical Bone

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To determine the effect of estrogen receptors (ER) $\alpha$ and $\beta$ on bones’ adaptive response to loading, we subjected the right tibiae of mice lacking ER$\alpha$ or ER$\beta$ activity to either axial loading or to disuse. Adaptive changes in architecture were assessed by comparing differences between the right (treated) and left (control) tibiae in these genotypes as assessed by microcomputed tomography. In female ER$\alpha^{-/-}$ mice, the net-osteogenic response to loading was lower in cortical bone compared with their wild-type littermates (11.2 vs. 20.9% in ER$\alpha^{+/+}$), but it was higher in both cortical and cancellous bone of male ER$\alpha^{-/-}$ mice (cortical 20.0 vs. 4.6% in ER$\alpha^{+/+}$; cancellous 30.0 vs. 5.3% in ER$\alpha^{+/+}$, $P < 0.05$). In ER$\beta^{-/-}$ male and female mice, the net-osteogenic response to loading was higher in cortical bone (males 10.9 vs. 3.9% in ER$\beta^{+/+}$; females 18.5 vs. 15.8% in ER$\beta^{+/+}$, $P < 0.05$) but no different from controls in cancellous bone. The bone loss in response to disuse was less in cancellous bone of ER$\alpha^{-/-}$ mice than in controls (−15.9 vs. −21.3%, respectively, $P < 0.05$) but no different at any other site or between any other groups. Our conclusion is that functional ER$\alpha$ enhances the net-osteogenic response to loading in cortical but not cancellous bone in female mice but reduces it in males. ER$\beta$ decreases the response to loading in cortical bone of males and females but has no effect in cancellous bone. Bone loss due to disuse in cortical bone is unaffected by ER status, but in cancellous bone, functional ER$\alpha$ contributes to greater disuse-related bone loss. *(Endocrinology 153: 2254–2266, 2012)*

The skeleton meets its responsibilities to provide structures sufficiently strong to withstand loading without fracture or undue accumulation of microdamage through a process of functional adaptation. This process is characterized by adjustment of bone mass and architecture in response to customary functional loading. The result of this adaptability is most readily seen where functional loading differs widely from normal such as high-impact sporting activities at one extreme and paraplegia or weightlessness at the other (1). However, for the overwhelming majority of the population, normality of loading is reflected by normality of skeletal mass and architecture (2–4).

The most widespread failure of functional adaptation to maintain a functionally appropriate match between bone loading and bone architecture is probably the decline in bone mass that occurs despite continued functional loading in age-related and postmenopausal osteoporosis. The result of this bone loss is an increase in the incidence of fragility fractures (5). In women, the association between the menopause and bone loss leading to osteoporosis has always suggested a causal relationship with the withdrawal of estrogens (6). However, the precise mechanisms involved have not yet been satisfactorily defined.

Estrogens are a class of steroid hormones that produce their actions primarily through two estrogen receptors (ER) $\alpha$ and $\beta$, which are members of the nuclear receptor superfamily encoded for by two distinct genes (7). The link between these receptors and bones’ adaptive response to loading in age-related and postmenopausal osteoporosis.
loading was first suggested by Damien et al. (8, 9), who showed that the proliferative response of osteoblasts to short exposure of mechanical strain in vitro was blocked by the ER modifiers ICI 182,780 and tamoxifen. Their inference that ER was involved in functional adaptation to loading was supported by their subsequent finding that in vivo, the osteogenic response to loading was three times less in female mice lacking ERα than in their wild-type (WT) littermates (10, 11). Osteoblast-like cells extracted from ERα−/− mice show a significantly lower proliferative response to strain (10) and ERK activation (12) compared with cells from WT animals. The number of genes differentially regulated in the 24 h after in vivo loading is also dramatically reduced in the bones of female mice lacking ERα activity compared with WT controls (13). The participation of ERα in bone cell response to loading does not require the presence of estrogens (14, 15), although estrogens are responsible for regulating ER number (16). In humans, variations in ERα gene expression (ESR1) are associated with bone mineral density, risk of osteoporotic fracture, and the responsiveness to exercise (17–26).

Although the greatest attention has been paid to the action of ERα, there is evidence that ERβ also influences loading-related responsiveness, in this case acting as an inhibitor to bones’ osteogenic response to loading (27). Thus, ERβ inhibits, whereas ERα enhances, gene-regulation in response to estrogens (28), and the influence of ERβ on bone mineral density in humans has been well documented. Single-nucleotide polymorphisms of the estrogen receptor β gene (ESR2) are associated with lower bone mineral density and a higher risk of osteoporosis (18, 19, 21, 29–34).

The experiments reported here were designed to address four deficiencies in our knowledge following the previous in vivo loading experiment by Lee et al. (10); 1) these authors used the ERα−/− mice developed by Korach and colleagues (35) that continue to express a 56-kDa fragment of the ER that could have some activity (36); 2) they only used females; 3) they only used a single load magnitude; and 4) they only measured the response in cortical bone. We therefore examined the response to graded loads and to disuse in cortical and cancellous regions of bone in both males and females of the two groups of mice derived by Dupont et al. (37) in which there is no remnant of either ERα or ERβ.

Materials and Methods

Animal model

Two mouse colonies were used in these studies: the ERα and ERβ genes deleted and backcrossed onto the same mouse strain (C57BL/6) (37). In both colonies, male and female heterozygotes were mated resulting in ERα−/−, ERα−/+, and WT (ERα+/+) mice in the ERα colony, and ERβ−/− and WT (ERβ+/+) offspring in the ERβ colony. Thus, besides the possible partial or full deletion of ERα or ERβ, all mice shared the same genetic background. At 3 wk of age, genotyping of ear DNA was performed by PCR using the following primers for ERα: P1 (5′-TTGCCCAGATAAACAACTAT-3′), P2 (5′-ATTGTCTTCITTCGACAC-3′), and P3 (5′-GGCATACCATCTCCTGGAGTCT-3′). The size of P1-P2 and P1-P3 fragments from WT mice is 364 bp and 889 bp, respectively, and that of the P1-P3 fragment from the ERα allele is 359 bp. For the ERβ colony, the following primers were used: P1 (5′-TATCCTAGCTCTGGGAAGGC-3′), P2 (5′-ACATTATATCGATCATCTCTGC-3′), and P3 (5′-AAGCGCATGCTCCGACTGC-3′), which yield a 381-bp fragment for WT mice (P1 and P2 primers), a 237-bp fragment for homozygous mutants (primers P1 and P3), and both fragments for heterozygotes.

The mice were housed up to five per cage and provided standard mouse chow and water ad libitum throughout the study. When they reached 17 wk of age, the mice either underwent sciatic neurectomy or were subjected to 2 wk of mechanical loading of the right tibia as described below. At 19 wk of age, they were euthanized and their tibia dissected and stored in 70% ethanol. All procedures were conducted in accordance with the Institutional Animal Care and Home Office, United Kingdom, guidelines and approved by the ethics committee of the Royal Veterinary College.

Strain measurement during dynamic axial loading

The mechanical strain at the proximal, medial surface of the tibia (37% of bone length from the proximal end) was measured postmortem with strain gauges in four or fewer male and female WT (ERα+/+, ERβ+/+), ERα−/−, ERα−/+, and ERβ−/− mice. Their previously frozen hindquarters were brought to room temperature in saline over 24 h and soft tissue dissected away to expose the medial surface of the tibia. A single element strain gauge (EA-06-015DJ-120; Vishay Measurement Group, Raleigh, NC) was bonded to the tibia in longitudinal alignment using cyanoacrylate adhesive. The tibia was then loaded in axial compression using the same peak loads as those used for in vivo loading using the same servo-hydraulic machine (Dartec HC10; Zwick Roell, Herefordshire, UK).

This calibration experiment showed a strong linear correlation between peak compressive force and longitudinal strain at the medial surface of the tibia for ERα−/−, ERα−/+, ERβ−/−, and WT (ERα+/+, ERβ+/+) mice. The magnitudes of load used for the loading experiment in vivo were selected to apply sufficient strain to engender adaptive change in bone architecture without causing damage to bone, joints, or skin through which the loads were applied.

In vivo tibia loading protocol

Eight male and female WT (ERα+/+, ERβ+/+) and ERα−/−, ERα−/+, ERβ−/− mice were randomly assigned to a high, medium, low, or very low mechanical loading group ranging in magnitude from −7 to −16.5 N. While under oxygen and halothane anesthesia (Merial, Dublin, Ireland), the right tibia from each group of mice was axially loaded for 40 cycles/d using a trapezoid waveform, with 14.9 sec rest in between each cycle, on 3 alternate days in a week for 2 consecutive weeks. When an axial force is applied to the tibia, the bone bends in the medial-lateral(direction).
direction, resulting in compression of the medial surface and tension on the lateral surface. The region of greatest new bone formation occurs in the proximal half (38). The left tibia was not loaded and used as a control (14). All mice were allowed normal cage activity in between loading periods.

**Disuse by sciatic neurectomy**

At 17 wk of age, seven to 11 male and female WT (ERα+/+, ERβ+/+) and ERα−/−, ERα+/−, and ERβ−/− mice underwent right unilateral sciatic neurectomy to produce disuse of the hind limb on that side. The mice were anesthetized using halothane and oxygen, and via a dorsal approach, a 3-mm section was excised from the right sciatic nerve. The wound was sutured, and after recovery, mice soon returned to normal cage activity, albeit with a paralyzed right hind limb. The left limb served as a normally functioning control. At 19 wk of age, the mice were euthanized, and both tibiae were extracted and stored in 70% alcohol.

**Microcomputed tomography (micro-CT)**

The entire tibiae were scanned *ex vivo* using micro-CT (Skyscan 1172; Kontich, Belgium) to measure cortical and trabecular bone parameters at a resolution of 4.9 μm × 4.9 μm. Bone length was measured to calculate 37% of bone length. This region corresponds to where the strains were measured and where we previously detected the highest strains and greatest changes in bone formation (38, 39). Hence, this is where changes to cortical bone architecture were assessed. A 0.49-mm-long segment (or 100 tomograms) was used for the analysis. For trabecular bone, a region starting immediately distal to the growth plate in the proximal metaphysis and extending 0.98 mm (or 200 tomograms) distally was analyzed. Histomorphometric analysis was performed by Skyscan software (CT-Analyzer version 1.5.1.3). For the analysis of trabecular bone, the cortical shell was excluded by operator-drawn regions of interest, and three-dimensional algorithms were used to determine the relevant parameters, which included: bone volume (BV) percentage [BV/trabecular volume (TV)], trabecular thickness, trabecular number, and trabecular space. For the analysis of cortical bone, two-dimensional computation was used, and parameters were determined for every one of the 100 tomograms and then averaged. These included total area (TA), cortical area (CA), and medullary area (MA). Coefficients of variation (CV) were determined by repeating a full scan (including repositioning), reconstruction, and analysis four times on the same sample. The CV of each parameter was determined as the ratio between the sd and the mean. The CV for relevant parameters were the following: tibia length 0.12%, BV/TV 1.57%, trabecular thickness 1.61%, CA 0.11%, and cortical thickness 0.29%.

**Statistical analysis**

A mixed-model analysis determined the effects of gender, genotype, and body weight on bone phenotype. Given significant differences in body weight between different genotypes, the effect of body weight was accounted for as a fixed covariate within each genotype. Bone phenotype values, corrected for body weight, are provided in Table 1. Pairwise comparisons were used to test for significant differences in bone phenotype within each gender and colony.

Percent side-to-side differences between the right (treated) and left (control) limbs were used throughout because it introduces some normalization for animal (and bone) size. Use of absolute measures would have been preferable to percentages if animals within each group could have been matched for size. Unfortunately, this was not possible.

The response to disuse was established by comparison between sciatic neurectomized and control limbs [(right − left)/left × 100]. An ANOVA determined the main effects of gender and

| TABLE 1. Bone phenotype of male and female ERα+/+, ERα+/−, ERα−/−, ERβ−/−/+, and ERβ−/− mice at 19 wk of age |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | F ERα+/+ | F ERα+/− | M ERα+/+ | M ERα−/− | M ERβ+/+ | M ERβ−/− |
| Weight (g) | 23.8 ± 0.5 | 26.6 ± 0.5 | 24.0 ± 0.5 | 26.7 ± 0.5 | 31.1 ± 0.5 | 33.8 ± 0.5 |
| Tibia Length (mm) | 17.98 ± 0.12 | 18.07 ± 0.07 | 18.11 ± 0.09 | 18.26 ± 0.07 | 18.33 ± 0.10 | 18.28 ± 0.05 |
| Cortical Bone | | | | | | |
| Cortical Area (mm²) | 0.972 ± 0.017 | 0.917 ± 0.010 | 0.892 ± 0.011 | 0.875 ± 0.012 | 1.000 ± 0.019 | 0.919 ± 0.14 |
| Total Area (mm²) | 1.577 ± 0.029 | 1.526 ± 0.016 | 1.508 ± 0.019 | 1.380 ± 0.017 | 1.664 ± 0.023 | 1.642 ± 0.023 |
| Medullary Area (mm²) | 0.604 ± 0.016 | 0.609 ± 0.009 | 0.616 ± 0.10 | 0.502 ± 0.011 | 0.651 ± 0.012 | 0.696 ± 0.015 |
| Cancellous Bone | | | | | | |
| BV/TV (%) | 9.836 ± 0.614 | 11.546 ± 0.321 | 13.582 ± 0.376 | 8.194 ± 0.426 | 10.950 ± 0.399 | 12.332 ± 0.376 |
| Th. Thickness (mm) | 0.051 ± 0.001 | 0.050 ± 0.001 | 0.044 ± 0.001 | 0.051 ± 0.001 | 0.051 ± 0.001 | 0.044 ± 0.001 |
| Th. Number (mm⁻³) | 1.903 ± 0.128 | 2.307 ± 0.067 | 3.127 ± 0.078 | 1.620 ± 0.087 | 2.013 ± 0.081 | 3.209 ± 0.079 |
| Th. Space (mm) | 0.287 ± 0.011 | 0.249 ± 0.001 | 0.182 ± 0.007 | 0.313 ± 0.009 | 0.278 ± 0.008 | 0.167 ± 0.005 |

Pairwise comparisons were used to test for significant differences in bone phenotype within each gender and colony. Only significant differences between ERα+/−, ERα−/−, and ERβ−/− mice and their WT littermates are shown for simplicity. Data shown are the mean ± SE. Tb, Trabecular.

*P < 0.05 to <0.001.*
genotype and any interaction between these on the effects of disuse. When a significant main effect was detected (P < 0.05), a Bonferroni post hoc analysis was undertaken. All data were analyzed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL). For significant interactions between gender and genotype, a Bonferroni post hoc analysis was conducted using SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC).

To assess whether any factors predicted the response to loading, the residuals of several variables were compared using the residual means squared model. In cortical bone, body weight gave the greatest and total bone area in the left limb the smallest residual values, thus indicating a better fit. This supports the assumption that the same load, in newtons, would impose a smaller strain on a larger bone than on a smaller bone and hence would predict the magnitude of the osteogenic response. As such, the percentage change in TA, MA, and CA was not adjusted. One-way ANOVA was used to assess the effects of gender and genotype on the percent changes. For each group of mice, we used the mixed-model procedure, with strain as a covariate to establish the presence of genotype-strain interactions. A significant genotype-strain interaction for any parameter may be due to different increases in the response at different magnitudes of strain (a change in the slope of the dose-response curve). When significant interactions were detected, a contrast analysis in SAS for Windows version 9.2 was performed to establish whether the slope of the line was significantly different from zero (indicating a statistically significant dose-response) and whether it was significantly different from the other groups. All tests were considered significant at P < 0.05.

**Results**

**Basal phenotype**

ERα and ERβ expression significantly influenced body weight and bone length (Table 1). Compared with their WT littermates, male and female ERα−/− mice were heavier (+8.7 to 31.1% vs. ERα+/+, P < 0.05 to <0.001) as were female mice lacking ERβ (+11.2% vs. ERβ+/+, P < 0.01). Bone length was greater in male ERβ−/− mice than in their WT littermates (+1.0% vs. ERβ+/+, P < 0.05), but it was not different in other mice compared with their WT littermates.

Genotype also influenced cortical and cancellous bone phenotype, even after it was corrected for the effect of body weight nested within each genotype. For cortical bone, the post hoc analysis of these corrected values (Table 2).

**TABLE 2.** Percent change in bone size of the sciatric neuromized limbs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CORTICAL BONE</th>
<th>CANCELLOUS BONE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cortical Area (mm²)</td>
</tr>
<tr>
<td>ERα−/−</td>
<td>N=8</td>
<td>0.935 ± 0.025</td>
</tr>
<tr>
<td>ERα+/+</td>
<td>N=9</td>
<td>0.925 ± 0.011</td>
</tr>
<tr>
<td>ERβ−/−</td>
<td>N=7</td>
<td>0.789 ± 0.013</td>
</tr>
<tr>
<td>ERβ+/+</td>
<td>N=9</td>
<td>0.858 ± 0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.107 ± 0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.035 ± 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.961 ± 0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.922 ± 0.041</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.008 ± 0.017</td>
</tr>
<tr>
<td>Gender</td>
<td>Genotype</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERα−/−</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERα+/+</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERβ−/−</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERβ+/+</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

This was calculated by comparing bone size in the sciatric neuromized (SN) to the contralateral control limbs (C) excised and scanned using micro-CT 2 wk after surgery [SN – C]/C × 100]. ANOVA was used to assess the effects of gender and genotype on the percent changes. Paired t tests tested for significant bone loss on the sciatric neuromized limb compared with the control limb. Data shown are the mean ± SE. NS, Not significant; Tb, trabecular.

P < 0.05. NS <0.001.
showed that in the ERα colony, the tibiae of female ERα−/− mice had a smaller CA compared with their female WT littermates (−8.2% vs. ERα+/+, P < 0.05). Male ERα−/− mice had a smaller TA and MA compared with their male WT littermates (TA −14.8% and MA −12.1% vs. ERα+/+, P < 0.001), but this did not equate to a difference in CA. Male and female ERα+/− mice showed a bone phenotype in between that of their respective ERα−/− mice and WT controls. In the ERβ colony, no differences in cortical bone size were detected between the tibiae of male and female ERβ−/− mice and their respective WT littermates.

In cancellous bone, post hoc analysis showed that the tibiae of female ERα−/− mice had a higher BV/TV compared with their WT littermates (+38.1% vs. ERα+/+, P < 0.001) due to higher trabecular number and a decrease in trabecular space (P < 0.05 to <0.001). The tibiae of female ERα+/− mice showed a cancellous bone phenotype in between that of female ERα−/− and their WT controls. In males, there was no difference in cancellous bone phenotype between ERα−/− and ERα+/+ or ERα+/− mice. In the

Disuse

Two weeks of disuse, induced by sciatic neurectomy, resulted in a significant decrease in CA, BV/TV, trabecular thickness, and trabecular number in the tibiae of all eight groups of mice (Table 2). The magnitude of loss was significantly influenced by genotype (P < 0.05 to <0.001). Surprisingly, no difference in the loss of CA was detected between ERα−/−, ERα+/−, and ERβ−/− mice and their WT controls (Fig. 1), although WT mice of the ERβ colony did lose more CA than those in the ERα colony (P < 0.001). The only gender-genotype interaction was for change in MA; however, the post hoc comparisons did not indicate any significant difference between the male and female ERα−/−, ERα+/−, and ERβ−/− mice and their re-

FIG. 1. Representative micro-CT images of the tibia demonstrating changes in CA in response to 2 wk of high strains of mechanical loading or disuse in male and female ERα+/+ and ERα−/− mice. The location of the cortical and cancellous bone sections analysed by micro-CT are indicated.
spective WT controls. Therefore, in male and female mice, cortical bone loss in response to disuse was not influenced by the absence of either ERα or ERβ.

In contrast, in cancellous bone, genotype influenced the degree of bone loss. The reduction in trabecular thickness was lower in ERα−/− mice compared with their WT controls and heterozygote ERα−/+ mice (−15.9% vs. −21.3% in ERα+/+ and −21.8% in ERα−/−, P < 0.05) (data for each genotype incorporating both males and females are not shown in Table 1), although this was insufficient to influence the reduction in BV/TV between these groups of mice. No difference in cancellous bone loss was detected between ERβ−/− mice and their WT littermates, nor were any gender-genotype interactions detected in cancellous bone. Thus, the absence of ERα but not ERβ reduces the degree of cancellous bone loss due to disuse in males and females.

**Loading**

Two weeks of the mechanical loading regimen producing very low to high physiological levels of peak strain (1520–3905 µε), on the medial side of the tibia at 37% of bone length from the proximal end, were associated with significant increases in CA in all groups of mice (P < 0.05 to <0.001) (Table 3). Slopes reflecting the relationship between change in strain and bone architecture were assessed in male and female mice within each genotype. A slope significantly different from zero reflected a dose-response relationship between increasing strain and the change in bone architecture (Tables 4 and 5, in bold).

All female mice from the ERα and ERβ colony showed significant dose-response relationships in CA and TA (P < 0.05 to <0.001) (in bold, Table 4). In the ERα colony, the gradients of the slopes were not significantly different between ERα−/− mice and their WT controls. However, the size of the net-osteogenic response was lower in ERα−/−.

**TABLE 3.** Percent change in the response to loading in all eight groups of mice at their corresponding magnitudes of strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>F ERα+/+</th>
<th>M ERα+/+</th>
<th>F ERα+/-</th>
<th>M ERα+/-</th>
<th>F ERα−/-</th>
<th>M ERα−/-</th>
<th>F ERβ+/+</th>
<th>M ERβ+/+</th>
<th>F ERβ+/-</th>
<th>M ERβ+/-</th>
<th>F ERβ−/-</th>
<th>M ERβ−/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Low Strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical Area %</td>
<td>2040µε (10.5N)</td>
<td>2770µε (12N)</td>
<td>2720µε (12.5N)</td>
<td>2150µε (10.2N)</td>
<td>1960µε (10N)</td>
<td>2420µε (10N)</td>
<td>3040µε (10N)</td>
<td>2070µε (10N)</td>
<td>3040µε (10N)</td>
<td>2070µε (10N)</td>
<td>3030µε (10N)</td>
<td>2070µε (10N)</td>
</tr>
<tr>
<td>Medullary Area %</td>
<td>2160µε (11N)</td>
<td>2800µε (15N)</td>
<td>2720µε (13N)</td>
<td>2180µε (11.5N)</td>
<td>2010µε (10N)</td>
<td>2600µε (15N)</td>
<td>3040µε (10N)</td>
<td>2070µε (10N)</td>
<td>3040µε (10N)</td>
<td>2070µε (10N)</td>
<td>3030µε (10N)</td>
<td>2070µε (10N)</td>
</tr>
</tbody>
</table>

**CORTICAL BONE**

| Low Strain |
| Cortical Area % | 2030µε (13N) | 2720µε (13N) | 2700µε (12N) | 2150µε (10.2N) | 1960µε (10N) | 2420µε (10N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |
| Medullary Area % | 2160µε (11N) | 2800µε (15N) | 2720µε (13N) | 2180µε (11.5N) | 2010µε (10N) | 2600µε (15N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |

**CORTICAL BONE**

| Medium Strain |
| Cortical Area % | 2040µε (12.5N) | 2770µε (12N) | 2720µε (12.5N) | 2150µε (10.2N) | 1960µε (10N) | 2420µε (10N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |
| Medullary Area % | 2160µε (11N) | 2800µε (15N) | 2720µε (13N) | 2180µε (11.5N) | 2010µε (10N) | 2600µε (15N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |

**CORTICAL BONE**

| High Strain |
| Cortical Area % | 2030µε (13N) | 2720µε (13N) | 2700µε (12N) | 2150µε (10.2N) | 1960µε (10N) | 2420µε (10N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |
| Medullary Area % | 2160µε (11N) | 2800µε (15N) | 2720µε (13N) | 2180µε (11.5N) | 2010µε (10N) | 2600µε (15N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |

**CORTICAL BONE**

| Cancellous Bone |
| Cortical Area % | 2030µε (13N) | 2720µε (13N) | 2700µε (12N) | 2150µε (10.2N) | 1960µε (10N) | 2420µε (10N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |
| Medullary Area % | 2160µε (11N) | 2800µε (15N) | 2720µε (13N) | 2180µε (11.5N) | 2010µε (10N) | 2600µε (15N) | 3040µε (10N) | 2070µε (10N) | 3040µε (10N) | 2070µε (10N) | 3030µε (10N) | 2070µε (10N) |

**CORTICAL BONE**

One-way t tests detected significant percent changes (shown in bold, P < 0.05 to <0.001). Unpaired t tests (ERβ colony) and ANOVA (ERα colony) were used to compare the percent changes between the different genotypes within each colony. Significant differences compared with WT mice are only shown for simplicity. Data shown are the mean ± se. Tb, Trabecular.

* P < 0.05 to <0.001.

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TABLE 4. The slope of the dose-response curves representing the responsiveness of each bone parameter to increasing mechanical strains in female mice for loading

<table>
<thead>
<tr>
<th></th>
<th>F ERα&lt;sup&gt;++&lt;/sup&gt;</th>
<th>F ERα&lt;sup&gt;+&lt;/sup&gt;</th>
<th>F ERα&lt;sup&gt;-&lt;/sup&gt;</th>
<th>F ERβ&lt;sup&gt;-&lt;/sup&gt;</th>
<th>F ERβ&lt;sup&gt;++&lt;/sup&gt;</th>
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<tr>
<td>CORTICAL BONE</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cortical Area %</td>
<td>0.013 ± 0.002</td>
<td>0.010 ± 0.002</td>
<td>0.011 ± 0.002</td>
<td>0.011 ± 0.002</td>
<td>0.016 ± 0.003</td>
</tr>
<tr>
<td>Total Area %</td>
<td>0.010 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.007 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Medullary Area %</td>
<td>0.000 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.002 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.000 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CANCELLOUS BONE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV %</td>
<td>0.017 ± 0.007</td>
<td>0.000 ± 0.008</td>
<td>0.015 ± 0.008</td>
<td>0.010 ± 0.009</td>
<td>0.013 ± 0.010</td>
</tr>
<tr>
<td>Tb.Thickness %</td>
<td>0.014 ± 0.004</td>
<td>0.015 ± 0.005</td>
<td>0.011 ± 0.005</td>
<td>0.003 ± 0.005</td>
<td>0.009 ± 0.006</td>
</tr>
<tr>
<td>Tb.Number %</td>
<td>0.002 ± 0.005</td>
<td>-0.005 ± 0.006</td>
<td>0.005 ± 0.006</td>
<td>0.003 ± 0.006</td>
<td>0.004 ± 0.008</td>
</tr>
<tr>
<td>Tb.Space %</td>
<td>-0.003 ± 0.004</td>
<td>0.007 ± 0.004</td>
<td>0.000 ± 0.004</td>
<td>0.008 ± 0.004</td>
<td>0.005 ± 0.005</td>
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</tbody>
</table>

A significant interaction between the strain and genotype was detected for TA and MA, indicating an effect of genotype on gradients of the slope (P < 0.05 to <0.001). Slopes in bold are significantly different from zero (P < 0.05 to <0.001). Data shown are the mean ± SE. Tb, Trabecular.

mice compared with their WT controls on the outer periostal surface at low up to high magnitudes of strain (CA –46.4 to –73.4% and TA –37.1 to –87.5% vs. ERα<sup>++</sup>, P < 0.05 to <0.001) but was greater on the inner endocortical surface (MA +70.6–186% vs. ERα<sup>++</sup>, P < 0.05 to <0.001) (Table 3 and Figs. 1 and 2A). Thus, in female mice, absence of ERα is associated with a reduced net-osteogenic response to loading on the periosteal surface of cortical bone.

In male mice, this situation was reversed because the net-osteogenic response to loading was greater in male ERα<sup>−/−</sup> mice than in their WT controls. Indeed, male ERα<sup>−/−</sup> mice showed the greatest net-osteogenic response in CA and TA in this whole study and their WT controls the least, with the ERα<sup>−/−</sup> mice in between (ERα<sup>−/−</sup> CA +339% and TA +353% vs. ERα<sup>++</sup>, P < 0.05 to <0.001) (Table 3 and Figs. 1 and 2C). No difference was detected on the inner endocortical surface (MA). A greater responsiveness to loading in male ERα<sup>−/−</sup> mice was also reflected by a steeper slope for CA and TA compared with their WT controls (P < 0.01) (Table 5). In these controls, the slopes were not significantly different from zero (Table 5).

Female ERβ<sup>−/−</sup> mice and their WT controls showed no difference in the slope of their net-osteogenic response to loading in cortical bone, but the magnitude of this response was greater in ERβ<sup>−/−</sup> mice compared with their WT littermates on the outer periosteal surface at high strains (CA +17.1% and TA +11.9% vs. ERβ<sup>++</sup>) but reduced on the inner endocortical surface (MA –54.1% vs. ERβ<sup>++</sup>) (P < 0.05 to <0.01) (Tables 3 and 4). In males, the ERβ<sup>−/−</sup> mice also showed a greater net-osteogenic response in CA and TA compared with their WT controls (CA +179.0% and TA +321.0% vs. ERβ<sup>++</sup>, P < 0.001) and a steeper dose-response relationship (P < 0.05 to <0.01) (Tables 3 and 5 and Fig. 2D).

In cancellous bone, exposure to low and high magnitudes of physiological strain significantly increased trabecular thickness in all groups of mice except male WT mice from the ERα colony (P < 0.05 to <0.001) (Table 3). This translated to a significant dose-response relationship in female ERα<sup>−/−</sup>, ERα<sup>+/−</sup>, and ERα<sup>++/−</sup> mice and male ERα<sup>++/−</sup> mice (in bold, Tables 4 and 5). In the ERα colony, neither the slope nor the magnitude of the loading response was significantly different between female ERα<sup>−/−</sup>, ERα<sup>+/−</sup>, and their WT controls (Tables 3 and 4 and Fig. 3A). In contrast, male ERα<sup>−/−</sup> and ERα<sup>++/−</sup> mice showed a greater net-osteogenic response in trabecular thickness compared with their WT controls (+273.4–363.8% ERα<sup>−/−</sup> vs. ERα<sup>++/−</sup>, respectively, P < 0.05 to <0.001) (Table 3 and Fig. 3C); however, this did not translate to a difference in the gradient of the slope between these mice (Table 5). In the ERβ colony, no difference was detected in the slope or the magnitude of the loading response between male and female ERβ<sup>−/−</sup> mice and their WT controls (Tables 3–5 and Fig. 3, B and D).

### Discussion

The overall inferences from this study are as follows: 1) In female mice, absence of ERα is associated with a lower
The net-osteogenic response to loading in cortical but not cancellous bone; 2) in contrast, in males, absence of ERα is associated with a higher net-osteogenic response to loading in both cortical and cancellous bone; 3) absence of ERβ is associated with a higher net-osteogenic response to loading in males and females in cortical but not cancellous bone; 4) absence of ERα has no effect on the amount of bone loss induced by disuse in cortical bone but is associated with lower cancellous bone loss in both males and females; and 5) absence of ERβ has no effect on cortical or cancellous bone loss associated with disuse in males or females.

Loading the tibia over a 2-wk period allowed us to quantify structural changes in both cortical and cancellous bone compartments. Significant net-osteogenic responses were detected in the cortical bone of all groups of mice and in cancellous bone of all groups except male WT mice from the ERα colony. Female mice lacking functional ERα showed a lower net-osteogenic response to loading on the periosteal surface of cortical bone than their WT littermates. This supports the original finding by Lee et al. (10, 11) and similar findings by Callewaert et al. (40) and Wındahl et al. (15). The net-osteogenic response to loading in cancellous bone was no different in female ERα−/− mice than in their WT littermates, suggesting that in females the contribution to mecano-responsiveness by ERα is more important in cortical than in cancellous bone. This is consistent with reports in humans that ERα is more highly expressed in cortical than cancellous bone (41) but appears at variance with the substantial loss of cancellous bone, potentially due to decreased mecano-responsiveness, in women associated with estrogen withdrawal at the time of menopause.

The finding that the loading-related response in males lacking ERα activity was higher rather than lower than that in controls is particularly interesting. We are aware of only one other report of load-induced changes in male ERα−/− mice, and in this study, they showed a lower re-
response to loading than their WT littermates (40). This difference could be because these authors used the Korach knockout mouse (42) in which there is continued expression of truncated isoforms (35, 36, 43, 44) with some capacity to mediate the effects of estrogens (44) rather than the Chambon-derived knockout that fully and unambiguously lacks any ERα expression (37). The importance of different ERα isoform functions is highlighted in a recent report that mice expressing a variant unable to bind to DNA estrogen response elements, but retaining the ability to participate in protein-protein interactions, have a lower bone mass than complete ERα knockout mice (45).

Any attempt to explain the mechanisms underlying our in vivo data are essentially speculation because we have no knowledge of changes in the many factors, in addition to the ER, that could have influenced bone modeling and remodeling. However, it is not unlikely that the increased net-osteogenic response to loading in our male ERα knockout mice could be associated with changes in other signaling pathways. The loss of the androgen receptor (AR) (40), ERα or ERβ are all associated with an increased response to loading in male mice. What compensatory mechanisms are activated in each of these cases and whether such compensation contributes to the maintenance of bone mass in ageing human males relative to females, remains to be elucidated.

The importance of ERβ on the skeleton is less well studied than that of ERα. In mouse bone, ERβ is reported to oppose the activity of ERα in response to estrogens (28). In male and female mice, we found the response to loading in the cortical bone at high strains was greater in ERα+/− mice than their WT littermates, but there was no difference in the size of this response in cancellous bone between these mice. An enhanced cortical bone’s response to loading has previously been reported in the ulna-cortex of female ERβ+/− mice (27). Extrapolating to the normal situation suggests that ERβ has no effect on mechano-

FIG. 3. Percent difference in BV/TV between right (loaded or disuse) and left (control) limbs in response to disuse induced by sciatic neurectomy (0 με) and varying magnitudes of strain engendered by in vivo axial loading in female and male mice with the ERα+/−, ERα−/−, ERα+/− (A and C), and ERβ+/− and ERβ−/− mice (B and D) backgrounds. The plotted line reflects the dose-response to loading in each group of mice, and the equations of the line are provided. Significant strain-genotype interactions indicated that the slopes were different between the genotypes (Tables 4 and 5). Unpaired-sample t tests compared the percent change in ERα+/−, ERα−/−, and ERβ−/− with their WT littermates at similar magnitudes of strain (Table 3). *, P < 0.05 to <0.001 vs. WT.
transduction in cancellous bone but a suppressive effect in cortical bone.

We had expected that differences in the net-osteogenic response to loading between the mice would have been reflected in their basal phenotype. This, however, was evident only in female ERα−/− mice, which showed a lower response to loading and corresponding smaller cortical bone volume in their basal bone phenotype. The finding that the net-osteogenic response to loading was significantly different at high but not lower strains highlights the risk of drawing potentially erroneous conclusions from experiments in which only a single load is used or in which these loads are insufficient to engender divergent responses.

The skeletons of the mice we used in this study were essentially mature. We would expect that the changes we report in both loading-related increases and decreases in bone mass would be greater during growth as previously reported in rats (46). However, despite potential ER-mediated influences on bone architecture derived from their interaction with estrogens and growth factors.

The role of estrogens in bone growth has long been recognized in females but more recently in males (47–49). Our data suggest that signaling via ERα, but not ERβ, is required for normal cortical bone growth in males and females. This has been reported elsewhere (50–55). The lack of a stimulatory role of ERβ on cortical bone growth could be explained by its ability to antagonize the activity of ERα (28). Our data also suggest that signaling via ERα and -β is required for normal cancellous bone growth only in females, not in males. This has previously been reported by Sims et al. (54) and Windahl (50). There does not appear to be any obvious suggestion for why ER should not be important for cancellous bone development in male mice because in humans, estrogens are clearly important in both males and females (56). It is possible that testosterone may be more important than estrogens in male mice, as has been found in the cancellous bone of pubertal boys (56, 57) and that signaling via the AR may compensate for the loss of ER. Callewaert et al. reported that AR but not ER are the mediators of the effects of sex steroids on cancellous bone mass in male mice (40), and osteoblast-specific AR knockout mice show a cancellous, but not a cortical, bone phenotype (58, 59). These conflicting findings highlight our poor understanding of the consequences of global deletion of either ER and that the role of AR requires further investigation.

The effect of 2 wk sciatic neurectomy-induced bone loss was apparent in measures of CA and cancellous bone volume in all groups of mice. The magnitude of this loss was, however, influenced only by ERα and not ERβ. Absence of ERα significantly reduced cancellous bone loss in both male and female mice, suggesting that in the normal situation, signaling via ERα facilitates cancellous bone loss in response to disuse but has no effect on cortical bone loss. We know of no other report on the effects of disuse in ERα−/− mice. In apparent contrast to our findings, the phenotype of osteoclast-specific ERα−/− mice suggests that normal ERα activity reduces cancellous bone loss in females by providing the mechanism through which estrogens reduce bone-resorbing osteoclast activity and number and increase osteoclast apoptosis (60, 61). Although these observations do not complement our findings in cancellous bone, the authors report a normal cortical bone phenotype in these mice, and this supports our inference that normal ERα activity does not influence cortical bone loss.

In the ERβ colony, we detected no difference in cortical or cancellous bone loss between ERβ−/− mice and their WT littermates. This suggests that normal ERβ activity plays an insignificant role in disuse-related bone resorption. This contrasts with preliminary findings by Castillo et al. (62) that female ERβ−/− mice show greater cancellous bone loss after 28 d of tail suspension compared with their WT littermates. It is possible that a longer period of disuse would have revealed differences between our mice or that tail suspension, which is known to induce stress-related responses in mice that alter bone metabolism, could have contributed to the contrasting results. Based on our findings alone, we propose that in the normal situation, ERβ has a minimal effect in cortical and cancellous bone loss.

Experiments to elucidate the mechanisms underlying the site-, gender-, and ER-specific responses we have identified could be addressed in mice with conditional ablation of ERα and ERβ activity in osteoblasts and osteocytes. This would eliminate confounding effects of elevated serum estradiol and testosterone, which would have been present in our study, and the opportunity for chronic compensation for the absence of each ER (37, 54, 60). Similar studies using mice that have one of the ER-binding domains deleted (activation function 1 and 2) or mice expressing a luciferase gene under control of an estrogen receptor element-containing promoter would be useful in determining the extent to which bone cell ER-mediated responses to loading are completely independent of a ligand.

The importance of ER in human bone is becoming ever more apparent. In the general population, polymorphisms in the ERα gene (ESR1) have been associated with bone mineral density (17–21), risk of vertebral fracture (23),
bone loss in men (24), and responsiveness to exercise (22, 25). Similarly, variations in ERβ gene (ESR2) expression are associated with low bone mineral density and an increased risk of fracture in both men and women (18, 19, 21, 29–34). Recent findings also show that ERα has a supportive role in a number of signaling pathways (13, 63). Thus, the development of new pharmacological therapies for osteoporosis that target the loading-related response (64) will need to take into account what role, if any, they may have on ER function and how it may influence the individual’s responsiveness to mechanical loading.

In conclusion, the absence of functional ERα decreases the net-osteogenic response to mechanical loading in the cortical but not cancellous bone of adult female mice. In contrast, absence of functional ERα increases this response in both cortical and cancellous bone of males. The absence of functional ERα has no effect on the bone loss associated with disuse in cortical bone of either male or female mice but is associated with reduced cancellous bone loss. The absence of functional ERβ increases the net-osteogenic response to loading of cortical bone in male and female mice but has no effect on that response in cancellous bone or in the response to disuse.

**Acknowledgments**

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