The adaptive response of bone to mechanical loading in female transgenic mice is deficient in the absence of oestrogen receptor-α and -β

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Abstract

Postmenopausal osteoporosis represents a failure of the response by which bone cells adapt bone mass and architecture to be sufficiently strong to withstand loading without fracture. To address why this failure should be associated with oestrogen withdrawal, we investigated the ulna's adaptive response to mechanical loading in adult female mice lacking oestrogen receptor-α ($ER\alpha^{-/-}$), those lacking oestrogen receptor-β ($ER\beta^{-/-}$) and their wild-type littermates. In wild-type mice, short periods of physiologic cyclic compressive loading of the ulna *in vivo* over a 2-week period stimulates new bone formation. In $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice this osteogenic response was respectively threefold and twofold less ($P<0{\cdot}05$). *In vitro*, primary cultures of osteoblast-like cells derived from these mice were subjected to a single short period of mechanical strain. Twenty-four hours after strain the number of wild-type cells was $61\pm25\%$ higher than in unstrained controls ($P<0{\cdot}05$), whereas in $ER\alpha^{-/-}$ cells there was no strain-related increase in cell number. However, the strain-related response of $ER\alpha^{-/-}$ cells could be partially rescued by transfection with functional human $ER\alpha$ ($P<0{\cdot}05$). $ER\beta^{-/-}$ cells showed a $125\pm40\%$ increase in cell number following strain. This was significantly greater than in wild types ($P<0{\cdot}05$).

These data support previous findings that functional $ER\alpha$ is required for the full osteogenic response to mechanical loading and particularly the stage of this response, which involves an increase in osteoblast number. $ER\beta$ appears to depress the $ER\alpha$-mediated strain-related increase in osteoblast number *in vitro,* but in female transgenic mice *in vivo* the constitutive absence of either $ER\alpha$ or $ER\beta$ appears to diminish the osteogenic response to loading.

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Introduction

Postmenopausal osteoporosis represents a failure of the normal homeostatic mechanisms by which functional load bearing establishes and maintains a sufficiently robust bone structure to withstand the forces of everyday activities without fracture (Lanyon & Skerry 2001). We hypothesise that this failure is due to a reduction in oestrogen receptor (ER) number and/or function in bone cells resulting from oestrogen deficiency.

It is currently believed that most of the functions of oestrogen in bone are mediated by $ER\alpha$ (Riggs *et al.* 2002). These include the regulation of osteoblast proliferation and differentiation, the synthesis of bone matrix proteins, the promotion of osteocyte survival, the suppression of bone resorption and the promotion of osteoclast apoptosis (Riggs *et al.* 2002). *In vitro* evidence suggests that $ER\alpha$ in bone cells is also involved in mediating responses to mechanical stimulation. The strain-related increase in cell number of monolayer cultures of rat and human osteoblast-like cells can be blocked with ER antagonists and enhanced by transfection with additional $ER\alpha$ (Damien *et al.* 1998, 2000, Zaman *et al.* 2000, Cheng *et al.* 2002). Moreover, mechanical strain stimulates $ER\alpha$ phosphorylation and oestrogen response element (ERE) activation via a pathway which is dependent on three well-established mediators of the *in vivo* osteogenic response of whole bones to mechanical loading: movement of calcium ions and the production of nitric oxide and prostaglandins (Zaman *et al.* 2000, Jessop *et al.* 2001, 2002).

Support for a reduction in ER number and function in bone cells in oestrogen-deficient states has been published by other workers. Bone biopsies from oestrogen-deficient women have fewer $ER\alpha$-positive osteocytes than those from oestrogen-replete women (Hoyland *et al.* 1999) and osteoblasts cultured from postmenopausal women are less responsive to oestrogen compared with osteoblasts from younger women in terms of oestrogen-induced collagen synthesis and ERE activation (Ankrom *et al.* 1998). In addition, reduced levels of $ER\alpha$ mRNA have been detected in trabecular bone following ovariectomy in rats (Lim *et al.* 1999).

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The inference that adaptive bone (re)modelling would be dependent upon functional ERα was supported by data from a pilot experiment *in vivo* (Lee *et al.* 2003), which showed that the loading response of ERα knockout (ERα$^{-/-}$) mice was threefold less than that in wild-type littermates. However, unravelling the effects of ER in bone cells is complicated by the presence of two ERs: ERα and ERβ. Both are present in osteoblasts but the action of ERβ in particular is currently uncertain (Riggs *et al.* 2002). Studies of ERβ knockout (ERβ$^{-/-}$) mice suggest that ERβ is not required for the protective effect of oestrogen on bone mass. The responses of the bones of young adult ERβ knockout (ERβ$^{-/-}$) female mice to ovariectomy and oestrogen-replacement therapy have been shown to be the same as those in wild types (Sims *et al.* 2002). However, 1-year-old female ERβ$^{-/-}$ mice have a low bone turnover and a high bone mineral density compared with wild types. This phenotype has been associated with higher ERα mRNA levels in the bones of ERβ$^{-/-}$ mice compared with wild types (Windahl *et al.* 2001). Therefore ERβ may have a repressive effect on ERα expression in bone and thus an inhibitory effect on the maintenance of an appropriate bone mass by oestrogen and mechanical loading.

The primary aim of the study reported here was to investigate the role of both ERα and ERβ in bone's adaptive response to mechanical loading. *In vivo* we did this by studying the osteogenic response of the ulna of ERα$^{-/-}$, ERβ$^{-/-}$ and wild-type mice to mechanical loading. We then cultured ERα$^{-/-}$, ERβ$^{-/-}$ and wild-type osteoblast-like cells from these mice and assessed *in vitro* changes in cell number following a period of mechanical strain. In addition, we assessed the effect of transfection of human ERα on the strain responsiveness of ERα$^{-/-}$ cells.

Materials and Methods

Mice

ERα knockout (ERα$^{-/-}$) and ERβ knockout (ERβ$^{-/-}$) mice were bred from two distinct breeding colonies. ERα$^{-/-}$ mice were homozygous for an insertional mutation in the first coding exon, exon 2, of the mouse ERα gene (Lubahn *et al.* 1993). ERβ$^{-/-}$ mice were homozygous for an insertional mutation in exon 3 of the mouse ERβ gene (Krege *et al.* 1998). The background strain of both colonies was C57Bl/6J. Knockout and wild-type littermates were bred from heterozygous breeding pairs. Hereafter ERα$^{+/+}$ and ERβ$^{+/+}$ mice represent the wild-type littermates of ERα$^{-/-}$ and ERβ$^{-/-}$ mice respectively.

Mice were genotyped by PCR analysis. Primers used for detection of the intact and disrupted ERα genes were 5′-AACTCGCCGGCTGCCACTTACCAT-3′ (ERα exon 2), 5′-CATCAGCGGGCTAGGCGACACG-3′
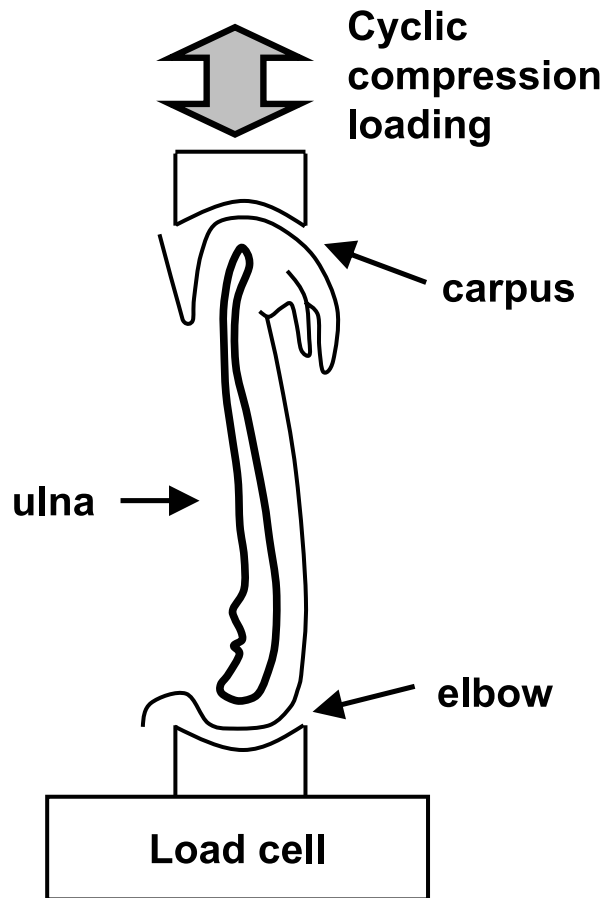

Figure 1 Diagram of the mouse ulna loading apparatus.

(ERα exon 2) (Vidal *et al.* 2000), 5′-GTGTTCCGGCTGTCAGCGCA-3′ (neomycin cassette) and 5′-GTCCTGATAGCGGTCCGCCA-3′ (neomycin cassette) (K S Korach, personal communication). Primers used for detection of the intact and disrupted ERβ genes were 5′-AGAATGTTGCACTGCCCCTGCTGC-3′ (ERβ intron 2), 5′-GGAGTAGAAACAAGCAATCCAGACATC-3′ (ERβ intron 3) and 5′-GCAGCCTCTGTTCCACATACACTTC-3′ (neomycin cassette) (Windahl *et al.* 1999).

Breeding colonies were maintained under standardized conditions, in a specific pathogen-free environment, with free access to water and a standard breeding or maintenance rodent diet as appropriate. All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and institutional guidelines.

Calibration of the *in vivo mechanical loading apparatus*

The technique for applying cyclic axial compression to the mouse ulna *in vivo* through the flexed elbow and flexed carpus has been published previously (Fig. 1) (Lee *et al.* 2002).

The forces required to produce the peak strains and strain rates previously recorded from the ulna during locomotion (Lee *et al.* 2002) were determined *ex vivo*, using four forelimbs from 20- to 24-week-old female mice from each of the following four groups: ERα$^{-/-}$ mice and their ERα$^{+/+}$ littermates; ERβ$^{-/-}$ mice and their ERβ$^{+/+}$ littermates. Strain gauges (EA-06–015 LA-120; Measurements Group UK Ltd, Basingstoke, Hamps, UK) were bonded to the lateral midshaft of each ulna and the forelimb positioned in the loading apparatus as shown in Fig. 1. The relationship between force and strain under axial compression loading was determined using linear regression analysis and slopes and intercepts were compared between genotypes using the Mann–Whitney test.

In vivo *loading*

The left ulnae of eight 20- to 24-week-old female ERα$^{-/-}$ mice and seven of their ERα$^{+/+}$ littermates were loaded by cyclic axial compression for 10 min on 3 alternate days each week for 2 weeks. In a separate experiment, the left ulnae of four 20- to 24-week-old female ERβ$^{-/-}$ mice and four of their ERβ$^{+/+}$ littermates were loaded using the same regimen. The right ulnae of these mice served as non-loaded paired controls. Loading sessions comprised 40 cycles of a trapezoidal waveform with a peak load of −3·4 Newtons (N), which engendered peak strains of 2800 microstrain at a maximum strain rate of 0·1/s at the lateral ulna midshaft. Following each load cycle there was a rest period of 14·9 s (Srinivasan *et al.* 2002).

Mice were anaesthetised for loading by intraperitoneal injection with a mixture of fentanyl citrate, fluanisone and sterile water and received 0·15 ml 0·9% sterile saline subcutaneously as routine anaesthetic fluid replacement therapy. Mice carried out normal activities between loading periods without signs of lameness. On day 15, blood samples were obtained from each mouse by intracardiac puncture under terminal inhalation halothane anaesthesia. Mice were killed by cervical dislocation.

Assessment of the in vivo *adaptive response to mechanical loading*

The ulna's response to loading was assessed histomorphometrically with the aid of double calcein labels (Sigma, Poole, Dorset, UK) administered at 7·5 mg/kg on days 3 and 12. Ulnae were harvested, cut transversely at the midshaft, fixed in neutral buffered formalin and embedded in methylmethacrylate as previously described (Mosley *et al.* 1997). Serial transverse sections were cut from the midshaft at 500 μm intervals, using a diamond sintered annular saw (Microslice II; Cambridge Instruments, Malvern, Worcs, UK). Computer images of the surface of each section were generated using the 488 nm argon laser and Plan-Neofluar 10 × objective lens (numerical

aperture 0·3) of a laser scanning confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK). Each pixel in these images measured 0·86 μm × 0·86 μm. Sections 3·5 mm distal to the midshaft were analysed in detail. This site lay within the region which showed the maximum loading response in previous experiments using adult CD1 mice (Lee *et al.* 2002). Quantitative histomorphometry was performed using the KS300 software (Imaging Associates Ltd, Thame, Oxon, UK), according to the recommendations of the American Society for Bone and Mineral Research (Parfitt *et al.* 1987) and as detailed previously (Lee *et al.* 2002).

Statistical differences between paired control and loaded ulnae for each genotype were determined using the paired *t*-test. ERα$^{-/-}$ and ERβ$^{-/-}$ ulnae were compared with their wild-type counterparts using the non-paired *t*-test. To control for differences between control ERα$^{+/+}$ and control ERα$^{-/-}$ ulnae and between control ERβ$^{+/+}$ and control ERβ$^{-/-}$ ulnae, 'loading-induced' values for each parameter were calculated by subtracting the value for each control ulna from that of its paired loaded ulna. Loading-induced values were compared between knockouts and their wild-type littermates using the non-paired *t*-test.

Primary osteoblast cultures

Osteoblast-like cells were isolated from cortical explants of the long bones of 6- to 10-week-old, male and female, ERα$^{-/-}$, ERβ$^{-/-}$ and wild-type mice using the cell outgrowth method as previously described (Jessop *et al.* 2002). All tissue culture reagents were obtained from Invitrogen unless otherwise stated. Briefly, all the soft tissues were removed from the long bones and their marrow cavities were flushed with phosphate-buffered saline (PBS). Long bones were then cut into small fragments and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal calf serum (FCS) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Bone fragments were incubated in 5% CO_2 at 37 °C for 21 days to achieve approximately 70% cell confluency. Media were replenished weekly.

On day 21, cells were washed in PBS and released with 0·05% trypsin and 0·02% EDTA for subculture. Only first passage cells were used for the following experiments. Sample cells were reserved at each extraction to confirm an osteoblastic phenotype. These cells expressed alkaline phosphatase, synthesised osteocalcin and formed mineralised nodules in long-term culture in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma) (Zaman *et al.* 1997).

Transfection and mechanical straining of osteoblast cultures

Four sets of primary osteoblast-like cell cultures were prepared for mechanical straining: ERα$^{-/-}$ cells,

ERβ$^{-/-}$ cells, wild-type cells and ERα$^{-/-}$ cells trans-fected with a functional human wild-type ERα expression vector, pRST7-ER (Tzukerman *et al.* 1994). All cells were seeded in monolayers onto sterile, tissue culture-treated, plastic strips (Nunc, Dossel, Germany) at a density of 80 000 cells per strip and incubated in DMEM con-taining 10% charcoal dextran-stripped FCS. The only exception was the ERα$^{-/-}$ cells for transfection, which were seeded at a density of 50 000 cells per strip.

ERα$^{-/-}$ cells were transiently transfected with pRST7-ER using Effectene (Qiagen) according to the manufacturer's instructions. One µg pRST7-ER vector was used per strip and the incubation period was 3 h. To determine transfection efficiency, cells were simul-taneously transfected with a *lac z* expression plasmid, at a concentration of 1 µg DNA per strip. β–Galactosidase activity was detected in fixed cells by histochemical staining (*lac z* staining kit; Promega) and the percentage of positive cells was calculated using phase contrast microscopy at a magnification of × 200.

Monolayer cell cultures were cyclically loaded by four-point bending for 10 min at a frequency of 1 Hz to peak strains of 3400 microstrain, using a custom designed loading apparatus as described previously (Zaman *et al.* 1997). Following loading, cultures were retained in the same culture medium and incubated for a further 24 h. The cells were then washed in PBS, fixed for 15 min in neutral-buffered formalin (BDH, Poole, Dorset, UK), washed twice in PBS and stored at 4 °C pending analysis. Control cultures were treated in the same way as strained cultures including being subjected to the same fluid perturbations, except they were not exposed to cyclic mechanical strain. Each experiment consisted of three strained and three control strips. Experiments were repeated three to five times.

Assessment of the proliferative response of osteoblasts to mechanical strain

The *in vitro* response of osteoblasts to mechanical strain was measured in terms of cell proliferation, by comparing the numbers of cells in strained cultures to those in non-strained control cultures by manual cell counting. To facilitate cell counting the nuclei of the fixed cells were stained with a fluorochrome, Hoechst 33258 (Sigma), using a modification of a previously described method (Rago *et al.* 1990). Briefly, fixed cells were washed with distilled water and incubated in 1 mg/ml Hoechst 33258 in TNE buffer (10 mM Tris, 1 mM EDTA and 2 M NaCl, pH 7·4) in the dark for 30 min. Cells were then blotted dry and mounted in glycerol. Cells were counted in 16 randomly chosen frames per strip using an ultraviolet microscope at × 4 magnification and the KS300 image analysis software (Imaging Associates Ltd). The mean number of cells per frame per strip was calculated. Cell number on strained and non-strained controls were
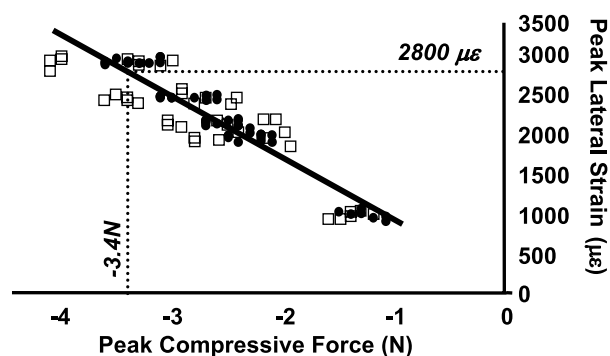

Figure 2 A scatter plot demonstrating the linear relationship between force and peak tension strain at the lateral ulna midshaft during cyclic axial compression loading *ex vivo* for ERα$^{+/+}$ (□) and ERα$^{-/-}$ (●) ulnae. This relationship was not significantly different between ERα$^{+/+}$ and ERα$^{-/-}$ ulnae. Ulnae were loaded *in vivo* using a peak compressive force of −3·4 N. (The corresponding scatter plot for ERβ$^{+/+}$ and ERβ$^{-/-}$ ulnae was very similar and is therefore not shown.) µε, microstrain.

compared using the paired *t*-test. Responses to mechanical strain were then expressed as the percentage difference in cell number between strained cultures and non-strained controls.

All statistical tests were carried out using SPSS10 (SPSS Inc., Chicago, IL, USA) and a 5% significance level. Data are presented as means ± S.E. unless otherwise stated.

Results

Absence of ERα or ERβ has no effect on stiffness under axial compression

ERα$^{-/-}$ ulnae (13·2 ± 0·02 mm, n=4) were 4% shorter (P<0·01) and straighter than ERα$^{+/+}$ ulnae (13·8 ± 0·01 mm, n=4). The length (and shape) of ERβ$^{-/-}$ ulnae (13·6 ± 0·17 mm, n=4) was not signifi-cantly different from ERβ$^{+/+}$ ulnae (13·7 ± 0·03 mm, n=4). *Ex vivo,* cyclic axial compression loading of each of these ulnae revealed a strong linear correlation (R^2>0·96, P<0·001) between peak compressive force and peak tension strain at the lateral ulna surface between 1·0 N and 4·0 N. No significant differences in the structural stiffness of the mouse ulna under cyclic axial compression loading were found between ERα$^{-/-}$ and ERα$^{+/+}$ mice and between ERβ$^{-/-}$ and ERβ$^{+/+}$ mice. Therefore data from knockout mice and their wild-type littermates were collated to predict the strain engendered at the lateral ulna midshaft under axial compression loading (Fig. 2). For ERα$^{-/-}$ and ERα$^{+/+}$ ulnae: peak lateral strain=(− 804·88 × peak compressive force)+104·56; R^2=0·86; P<0·001. For ERβ$^{-/-}$ and ERβ$^{+/+}$ ulnae: peak lateral strain=(− 730·25 × peak compressive force)+365·21; R^2=0·69; P<0·001. Therefore for both

Table 1 Ulna cortical areas and perimeters

	ERα$^{+/+}$ (*n*=7)	ERα$^{-/-}$ (*n*=8)	ERβ$^{+/+}$ (*n*=4)	ERβ$^{-/-}$ (*n*=4)
Cortical bone area (mm^2)	0·206 ± 0·005	0·231 ± 0·007	0·181 ± 0·006	0·180 ± 0·007
		P=0·021		n.s.
Marrow cavity area (mm^2)	0·024 ± 0·003	0·022 ± 0·002	0·017 ± 0·003	0·022 ± 0·005
		n.s.		n.s.
Periosteal perimeter (mm)	1·884 ± 0·032	1·987 ± 0·032	1·882 ± 0·028	1·812 ± 0·044
		P=0·042		n.s.
Endosteal perimeter (mm)	0·615 ± 0·034	0·598 ± 0·021	0·529 ± 0·053	0·587 ± 0·063
		n.s.		n.s.

Cortical areas and perimeters were measured 3·5 mm distal to the ulna midshaft in the non-loaded control ulnae.
P values represent comparisons between knockouts (ERα$^{-/-}$ and ERβ$^{-/-}$) and their wild-type littermates (ERα$^{+/+}$ and ERβ$^{+/+}$). n.s., *P*>0·05.

knockouts and their wild-type littermates a peak compressive force of −3·4 N resulted in a peak lateral strain of 2800 microstrain.

Histomorphometric comparisons between non-loaded control knockout and wild-type ulnae

Histomorphometric analysis of the non-loaded control ulnae for the *in vivo* loading experiment demonstrated small but significant differences between the baseline adult skeletal phenotype of ERα$^{-/-}$ and ERα$^{+/+}$ mice. There were no significant differences between the baseline adult skeletal phenotype of ERβ$^{-/-}$ and ERβ$^{+/+}$ mice.

Non-loaded control ERα$^{-/-}$ ulnae had a 12% greater cortical area and a 6% greater periosteal perimeter than non-loaded control ERα$^{+/+}$ ulnae, but there were no significant differences in marrow cavity area or endosteal perimeter between genotypes (Table 1). Periosteal modelling activity was barely detectable in all non-loaded control ulnae, as expected for adult mice with a mature skeleton (Sheng *et al.* 1999) (Fig. 3). Likewise, endosteal bone turnover was consistent with skeletal maturity in all non-loaded control ulnae, but endosteal mineralising surface and bone formation rate were significantly lower in ERα$^{-/-}$ ulnae compared with ERα$^{+/+}$ ulnae (*P*<0·050) (Fig. 4).

The osteogenic adaptive response to mechanical loading is threefold lower in ERα$^{-/-}$ mice compared with ERα$^{+/+}$ mice

Short periods of mechanical loading to peak strains of 2800 microstrain on 3 alternate days each week for 2 weeks stimulated periosteal and endosteal bone formation in the ulnae of both adult female ERα$^{-/-}$ and ERα$^{+/+}$ mice. In ERα$^{+/+}$ ulnae this resulted in an 8 ± 0·8% (*P*<0·001) increase in cortical area. However, in ERα$^{-/-}$ ulnae the loading-induced increase in cortical area was threefold less (*P*<0·001) than in ERα$^{+/+}$ ulnae resulting in only a 2·4 ± 0·8% (*P*<0·001) increase in cortical area (Fig. 5).

In both ERα$^{+/+}$ and ERα$^{-/-}$ ulnae, 80% of the loading-induced increase in cortical area was due to periosteal expansion and the remainder was due to endosteal new bone formation (Table 2). Both loading-induced periosteal and endosteal bone formation rates were 60% lower in ERα$^{-/-}$ ulnae compared with ERα$^{+/+}$ ulnae (*P*=0·001) (Figs 3 and 4). At the periosteum this was due to a 40% lower loading-induced increase in periosteal mineralising surface in ERα$^{-/-}$ ulnae compared with
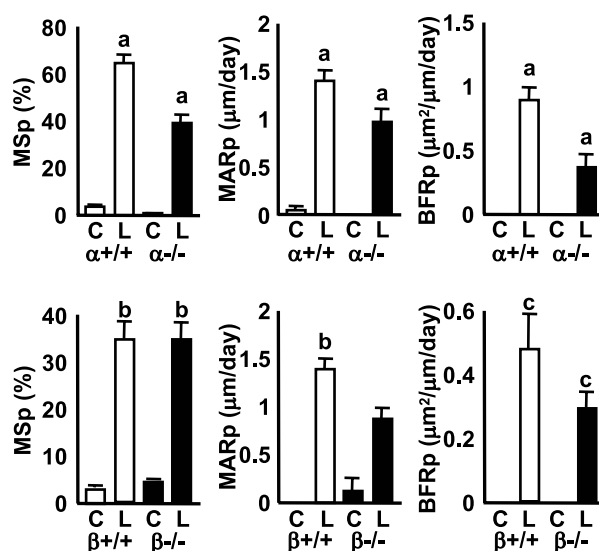
Figure 3 Histomorphometric analysis of the periosteal osteogenic response to mechanical loading in ERα$^{+/+}$ (α+/+), ERα$^{-/-}$ (α−/−), ERβ$^{+/+}$ (β+/+) and ERβ$^{-/-}$ (β−/−) ulnae. Periosteal mineralising surface (MSp), periosteal mineral apposition rate (MARp) and periosteal bone formation rate (BFRp) in loaded (L) and control (C) ulnae. a, *P*<0·001; b, *P*<0·010; c, *P*<0·050 for comparisons between control and loaded ulnae of the same genotype. Values are means ± S.E.
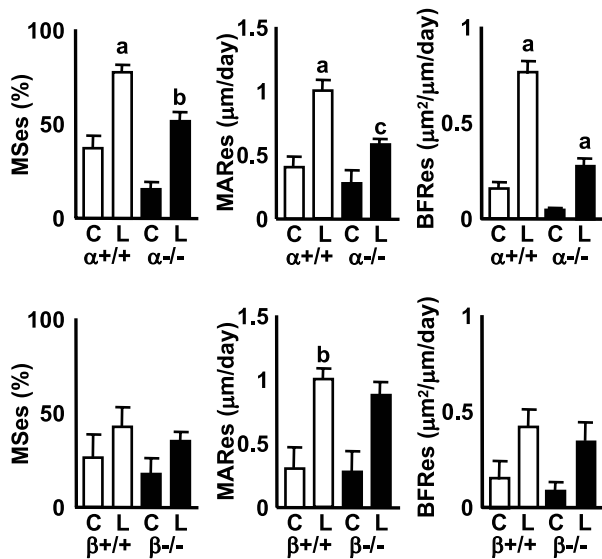
Figure 4 Histomorphometric analysis of the endosteal osteogenic response to mechanical loading in ER$\alpha^{+/+}$ (α+/+), ER$\alpha^{-/-}$ (α−/−), ER$\beta^{+/+}$ (β+/+) and ER$\beta^{-/-}$ (β−/−) ulnae. Endosteal mineralising surface (MSes), endosteal mineral apposition rate (MARes) and endosteal bone formation rate (BFRes) in loaded (L) and control (C) ulnae. a, $P<0.001$; b, $P<0.010$; c, $P<0.050$ for comparisons between control and loaded ulnae of the same genotype. Values are means ± S.E.

ER$\alpha^{+/+}$ ulnae ($P=0.001$) and at the endosteum this was due to a 50% lower loading-induced increase in endosteal mineral apposition rate ($P=0.023$).

The osteogenic adaptive response to mechanical loading is twofold lower in ER$\beta^{-/-}$ mice compared with ER$\beta^{+/+}$ mice

Short periods of mechanical loading to peak strains of 2800 microstrain on 3 alternate days each week for 2 weeks
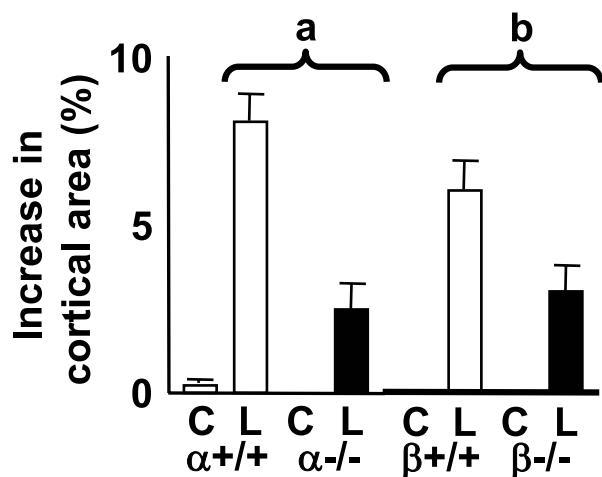

Figure 5 The loading-induced percentage increase in cortical area in ER$\alpha^{+/+}$ (α+/+), ER$\alpha^{-/-}$ (α−/−), ER$\beta^{+/+}$ (β+/+) and ER$\beta^{-/-}$ (β−/−) ulnae. C, control ulnae; L, loaded ulnae. a, $P<0.001$; b, $P<0.010$ for comparisons between knockout and wild-type littermates. Values are means ± S.E.

stimulated periosteal (but not endosteal) bone formation in the ulnae of both adult female ER$\beta^{-/-}$ and ER$\beta^{+/+}$ mice. In ER$\beta^{+/+}$ ulnae, this resulted in a $6 \pm 1.0\%$ ($P=0.007$) increase in cortical area. However, in ER$\beta^{-/-}$ ulnae, the loading-induced increase in cortical area was twofold less ($P=0.035$) than in ER$\beta^{+/+}$ ulnae resulting in only a $3.1 \pm 0.7\%$ ($P=0.017$) increase in cortical area (Fig. 5). This was associated with a 50% lower loading-induced increase in periosteal mineral apposition rate in ER$\beta^{-/-}$ ulnae compared with ER$\beta^{+/+}$ ulnae ($P=0.049$, Table 2).

There was no evidence of gross fractures, microfractures, bone resorption or intracortical remodelling in any of the loaded ulnae. The loading-induced osteogenic responses were therefore representative of physiological adaptation to an altered mechanical environment as demonstrated previously using the CD1 mouse (Lee *et al.* 2002).

The in vitro proliferative response to mechanical strain of osteoblast-like cells is absent in ER$\alpha^{-/-}$ cells, but enhanced in ER$\beta^{-/-}$ cells compared with wild-type cells

A 10-min period of mechanical strain resulted in a $61 \pm 25\%$ increase in cell number in osteoblast-like cells derived from ER$\alpha^{+/+}$ mice ($P=0.05$) over the following 24 h. The same treatment resulted in a $125 \pm 40\%$ increase in cell number in osteoblast-like cells derived from ER$\beta^{-/-}$ mice ($P=0.02$). Conversely, osteoblast-like cells derived from ER$\alpha^{-/-}$ mice showed no such increase in proliferation in response to mechanical strain, but rather experienced a $24 \pm 9\%$ decrease in cell number ($P=0.01$). However transfection of osteoblast-like cells derived from ER$\alpha^{-/-}$ mice with a functional wild-type ERα vector restored the proliferative response to mechanical strain, resulting in a mechanical strain-induced $48 \pm 8\%$ increase in cell number ($P=0.02$). This restoration was associated with a transfection efficiency of $20 \pm 2\%$.

Discussion

In vivo, the ulna of female mice lacking fully functional ERα but possessing functional ERβ have a threefold lower osteogenic response to mechanical loading than their wild-type littermates. This finding in ER$\alpha^{-/-}$ mice is consistent with the demonstration *in vitro* that ER$\alpha^{-/-}$ osteoblast-like cells fail to increase their number in response to mechanical strain, but that this response can be restored by transfection with functional human ERα. These findings support the concept that ERα is required for at least those components of bone's early adaptive response to mechanical loading, which involves increase in the number of osteoblasts and the formation of new bone.

Table 2 The effect of mechanical loading on cortical bone formation

	ERα$^{+/+}$ (n=7)		ERα$^{-/-}$ (n=8)	ERβ$^{+/+}$ (n=4)		ERβ$^{-/-}$ (n=4)
Loading-induced changes						
Periosteal						
New bone area (% of cortical bone area)	6·4 ± 0·7	P<0·001	2·0 ± 0·4	6·0 ± 1·0	P=0·036	3·0 ± 0·7
Mineralising surface (%)	61·5 ± 3·5	P=0·001	38·3 ± 3·7	31·3 ± 3·9	n.s.	29·7 ± 5·0
Mineral apposition rate (µm/day)	1·34 ± 0·14	n.s.	0·97 ± 0·17	1·40 ± 0·18	P=0·049	0·72 ± 0·26
Bone formation rate (µm²/µm per day)	0·90 ± 0·09	P=0·001	0·37 ± 0·06	0·49 ± 0·11	n.s.	0·30 ± 0·08
Endosteal						
New bone area (% of cortical bone area)	1·4 ± 0·2	P<0·001	0·4 ± 0·1	0·01 ± 0·00	n.s.	0·004 ± 0·002
Mineralising surface (%)	40·7 ± 4·5	n.s.	35·7 ± 8·2	17·8 ± 23·6	n.s.	18·8 ± 12·1
Mineral apposition rate (µm/day)	0·59 ± 0·07	P=0·023	0·30 ± 0·09	0·72 ± 0·14	n.s.	0·63 ± 0·26
Bone formation rate (µm²/µm per day)	0·61 ± 0·05	P<0·001	0·24 ± 0·04	0·29 ± 0·17	n.s.	0·26 ± 0·14

All parameters represent the loading-induced effect on bone formation 3·5 mm distal to the ulna midshaft and were calculated by subtracting the value for each control ulna from that of its paired loaded ulna.
P values represent comparisons between knockouts (ERα$^{-/-}$ and ERβ$^{-/-}$) and their wild-type littermates (ERα$^{+/+}$ and ERβ$^{+/+}$). n.s., *P*>0·05.

Two ERα mRNA splice variants, which could result in the translation of a 55 kDa protein and a 46 kDa protein, have been detected in tissues from ERα$^{-/-}$ mice (Couse *et al.* 1995, Pendaries *et al.* 2002, Shughrue *et al.* 2002). The 55 kDa protein has not been detected in wild-type tissues and there is no evidence to suggest that it is capable of mimicking the actions of the full-length ERα protein *in vivo* (Couse *et al.* 1995, Pendaries *et al.* 2002). The 46 kDa ERα isoform contains all the regions of the classical full-length ERα protein apart from the A/B domain (Pendaries *et al.* 2002). It has been shown to be capable of oestrogen-dependent ERE activation *in vitro* and *in vivo* and has been isolated from human primary osteoblasts (Metzger *et al.* 1995, Denger *et al.* 2001, Pendaries *et al.* 2002, Shughrue *et al.* 2002). The exact function of this protein in normal physiology has yet to be determined. Nonetheless, its possible presence does not detract from the findings of this study since it was clearly unable to fully compensate for the absence of the full-length ERα protein in this model of bone adaptation.

If ERα is required for bone's adaptive response to mechanical loading, then the simple absence of functional ERα should lead to a disuse bone phenotype. In this study, the ulnae of adult female ERα$^{-/-}$ mice were observed to be straighter than their wild-type counterparts. This is significant because mechanical loading is required for the development of a normal bone curvature (Chalmers & Ray 1962, Lanyon 1980, Rodriguez *et al.* 1988). The absence of a normal ulnar curvature in ERα$^{-/-}$ mice is

therefore consistent with an abnormal response to natural mechanical loading during growth. However, in other respects the structure of the ERα$^{-/-}$ ulnae does not suggest a disuse phenotype: these bones are shorter in length, with a greater cortical thickness and similar structural stiffness to wild-type ulnae (Vidal *et al.* 1999, 2000, Lindberg *et al.* 2001).

The absence of an uncomplicated disuse phenotype and the presence of a small *in vivo* osteogenic response to mechanical loading in ERα$^{-/-}$ mice may be due to a number of influences including high circulating oestrogen and testosterone concentrations (Couse & Korach 1999, Kousteni *et al.* 2001, Lindberg *et al.* 2002, Sims *et al.* 2002), low insulin-like growth factor concentrations (Lindberg *et al.* 2001) and possible compensatory effects of unopposed ERβ (Sims *et al.* 2002).

The *in vivo* and *in vitro* data from the ERβ$^{-/-}$ mice appear inconsistent with one another. *In vivo*, the ulna of female mice lacking fully functional ERβ but possessing functional ERα have a twofold lower osteogenic response to mechanical loading than their wild-type littermates. Conversely, *in vitro* ERβ$^{-/-}$ osteoblast-like cells show a greater increase in cell number in response to mechanical strain compared with wild-type cells. The data presented in this study do not permit us to explain this apparent discrepancy.

The *in vitro* data suggested that ERβ decreases while ERα enhances the strain-related increase in cell number in response to mechanical strain. This suggested that ERβ

either opposes the effects of ERα or suppresses the effects of ERα with respect to the ERα-mediated strain-related increase in osteoblast cell number. Such antagonism between ERα and ERβ has been described in previous *in vitro* studies. One such study using a mouse osteoblast cell line found that an increase in ERβ expression resulted in a suppression of oestrogen-dependent ERα-mediated activation of the AP1 transcriptional factor, c-fos (Ishibashi *et al.* 2001). Another study found that ERα and ERβ have opposite effects on AP1 transcription sites in breast cancer and uterine cell lines (Paech *et al.* 1997). However, the *in vivo* data suggested that the presence of ERβ as well as ERα enhances bone's osteogenic response to mechanical loading.

In our present study it may be that ERβ acts differently in the absence of ERα than in its presence (Sims *et al.* 2002). For example, their dual presence may result in the formation of heterodimers, which may have distinct actions compared with those of homodimers (Pettersson *et al.* 1997). However, this would not explain the difference in response between the *in vitro* and *in vivo* experiments presented, since the cells in the *in vitro* study were derived from the same population of mice as that used in the *in vivo* study. The ERβ$^{-/-}$ mouse used in this study is believed to be a true knockout and has not been shown to contain any mutant or non-mutant ERβ gene transcripts (Krege *et al.* 1998). Therefore the presence of such transcripts should not be involved in this response. However, secondary consequences of the absence of functional ERβ protein in other tissues may affect the *in vivo* responses of bone to mechanical loading, but may not be evident in pure cultures of osteoblast-like cells. In addition, the formation of new bone during a 2-week period of loading *in vivo* is likely to be a much more complex process compared with the increase in number of osteoblast-like cells in the 24 h after strain *in vitro*.

We can conclude from the results of this study that functional ER number is an important determinant of the capacity of bone to adapt to mechanical loading *in vivo*. Changes in functional ERα number may help to explain the aetiology of the structurally inappropriate bone loss, which occurs at and after the menopause in women and is associated with low available oestrogen levels in men. Our data are somewhat equivocal on the role of ERβ. The level of strain-related change in cell number *in vitro* suggested that ERβ opposes the effect of ERα. However, there was no evidence of any such inhibition being removed *in vivo* where the constitutive absence of either ERα or ERβ is associated with a smaller osteogenic response to loading than if both receptors were present.

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