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Osteocytes Use Estrogen Receptor α to Respond to Strain but Their ERα Content Is Regulated by Estrogen

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ABSTRACT: The role of mechanical strain and estrogen status in regulating ERα levels in bone cells was studied in female rats. OVX is associated with decreased ERα protein expression/osteocyte, whereas habitual strain and artificial loading has only a small but positive effect, except on the ulna's medial surface, where artificial loading stimulates reversal of resorption to formation.

Introduction: Osteoporosis is the most widespread failure of bones' ability to match their architectural strength to their habitual load bearing. In men and women, the severity of bone loss is associated with bioavailability of estrogen. This association could result from the estrogen receptor (ER) involvement in bone cells' adaptive response to loading.
Materials and Methods: In vivo semiquantitative analysis of the amount of ERα protein per osteocyte was performed in immuno-cytochemically stained sections from control and loaded rat ulna, as well as tibias of ovariectomy (OVX) and sham-operated female rats. In vitro, the effect of exogenous estrogen (10^{-8} M) and mechanical strain (3400 με, 1 Hz, 600 cycles) on the expression of ERα mRNA levels was assessed in ROS 17/2.8 cells in monolayers using real-time PCR and ER promoter activity. ERα translocation in response to exogenous estrogen and mechanical strain was assessed in both ROS 17/2.8 and MLO-Y4 cells.
Results: More than 90 percent of tibial osteocytes express ERα, the level/osteocyte being higher in cortical than cancellous bone. OVX is associated with decreased ERα protein expression/osteocyte, whereas in the ulna habitual strain and that caused by artificial loading had only a small but positive effect, except on the medial surface, where loading stimulates reversal of resorption to formation. In unstimulated osteocytes and osteoblasts in situ, and osteocyte-like and osteoblast-like cells in vitro, ERα is predominantly cytoplasmic. In vitro, both strain and estrogen stimulate transient ERα translocation to the nucleus and transient changes in ERα mRNA. Strain but not estrogen also induces discrete membrane localization of ERα.
Conclusions: Bone cells' responses to both strain and estrogen involve ERα, but only estrogen regulates its cellular concentration. This is consistent with the hypothesis that bone loss associated with estrogen deficiency is a consequence of reduction in ERα number/activity associated with lower estrogen concentration reducing the effectiveness of bone cells' anabolic response to strain.
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Key words: osteocytes, mechanical loading, estrogen, bone, osteoporosis

INTRODUCTION

The realization that estrogen receptor α (ERα) is involved in bone cells' adaptive response to mechanical strain[1,2] suggested the hypothesis that the rapid loss of bone that accompanies estrogen withdrawal is essentially similar in origin to that which accompanies the onset of disuse.[3] In disuse, the strain-related stimulus to positively balanced remodeling is reduced because of diminished loading. With estrogen withdrawal, the strain-related stimulus is reduced because it is processed less effectively because of estrogen concentration–dependent downregulation of the number or activity of ERs in the relevant bone cells. To validate this hypothesis, it is necessary (1) to substantiate ER's obligatory involvement in bone's adaptation to loading and (2) to show that the abundance and/or activity of ER in bone cells is regulated by estrogen and that this downregulation is not compensated for by upregulation arising from the increased strain accompanying bone loss.

A number of studies support ER's involvement in bone cells' adaptive responses to mechanical strain. In vivo, the osteogenic response to a short period of dynamic loading in $ERα^{-/-}$ mice is substantially less than in their $ERα^{+/+}$ or $ERα^{+/-}$ littermates.[4] As in the uterus, ERβ seems to modulate or oppose the effects of ERα.[5] Bone cells possessing ERα but lacking ERβ show a higher response to mechanical strain both in vitro[6] and in vivo[7] than cells with both ER isoforms. In vitro, osteoblast-like cells cultured from $ERα^{-/-}$ mice show a lower proliferative re-

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sponse to short periods of dynamic strain than those from either ERα$^{+/+}$ homozygotes or ERα$^{+/-}$ heterozygotes.[8] This lack of response in ERα$^{-/-}$ cells can be rescued by transfection of fully functional ERα but not by dysfunctional mutants.[8] In ROS 17/2.8 osteoblast-like cells, strain-related proliferation is blocked by the ER modulator ICI 182,780 and enhanced by transfection with additional ERα to produce ROS.SMER cells.[9] In rat primary osteoblast-like cells, both strain and estrogen stimulate phosphorylation of ERα by an extracellular signal-related kinase (ERK)-mediated mechanism[10] and upregulate activation of estrogen response elements (EREs), a response that is blocked by ICI 182,780.[9] Significantly, in female human primary osteoblast-like cells, and in the human TE85 cell line,[11] the proliferative responses to strain and estrogen are blocked by ICI 182,780 but not by tamoxifen.

In humans, the severity of bone loss, postmenopausally in women, and related to age in men, is related to bioavailability of estrogen.[12] However, there are scarce data from humans to discriminate between the effects on bone remodeling of ER activity as distinct from availability of estrogen. That the ERα mutant man should be profoundly osteoporotic[13] is consistent with ER's involvement in osteogenic responses to loading but his bone phenotype, like that of ERα$^{-/-}$ mice, may have been affected by high concentrations of estrogen acting independently of the ER. The phenotype of ERα mutation is similar to that of aromatase deficiency,[14,15] which is associated with reduced circulating estrogen. Aromatase deficiency in mice and men is associated with high levels of bone resorption and low bone mass.[16] Aromatase deficiency can be rescued by exogenous estrogen,[13] whereas the ERα null mutation cannot.[15] That exercise-related responsiveness in bone mass in adolescent girls should be associated with allelic variation in the *Pvu*II polymorphism of the ER further suggests involvement of the ER in the adaptive process to load bearing.[17]

This study was designed to investigate the effects in vivo of estrogen and mechanical loading on the ERα status of osteocytes. These are resident bone cells assumed to be involved in the earliest stages of strain-related control of (re)modeling. The presence and concentration of ERα was assessed by microdensitometric measurement of immunolocalized ERα in osteocytes of tibias in intact and ovariectomized (OVX) female rats and in the ulna of intact female rats subjected to short periods of artificial loading. These studies on the comparatively long term in vivo responses of osteocyte ERα to estrogen and bone loading were supported by studies in vitro on the dynamics of the effects of estrogen and strain on ERα within hours of treatment.

MATERIALS AND METHODS

In vivo loading

The left ulna of five female 230- to 250-g Sprague-Dawley rats (Charles River) were loaded nonsurgically on days 1, 3, 5, 8, 10, 12, and 15 of the experiment. The right ulna served as a nonloaded control. Axial loads were applied dynamically for 10 minutes at 2 Hz as a ramped square wave, producing a peak compressive strain of 3400 με at the bone's medial surface.[18,19] On day 16, the animals were killed using sodium pentobarbitone (Euthsate; Willows Francis Veterinary, Crawley, UK). These experiments were approved by the College's Ethics and Welfare Committee. Ulna were removed, dissected of soft tissue, and fixed in formalin (BDH). Previous studies showed that the largest osteogenic response occurred 2.5 mm distal to the midshaft.[18–21] Transverse serial sections were taken 2.2–2.7 mm distal to midshaft and ERα immunolocalized as described below.

Bilateral OVX

Sprague-Dawley rats (160–170 g) underwent OVX. Four weeks later, both OVX and sham-operated animals ($n = 5$) were killed by sodium pentobarbitone overdose. The tibias were removed, dissected of soft tissue, and fixed in formalin.

ERα immunohistochemistry

ERα was immunolocalized in decalcified, wax-embedded tibial and ulnar 8-μm sections using indirect immunoperoxidase.[22,23] Rabbit polyclonal MC-20 (0.2 μg/ml, shown not to cross-react with ERβ; Santa Cruz Biotechnology) and biotinylated anti-rabbit (Vector Laboratories) were used as primary and secondary antibodies, respectively. All antibodies were diluted in 0.1% BSA (Sigma) in PBS. Rabbit IgG and omission of primary antibody as well as the primary antibody preincubated with the blocking peptide (sc-542 P; Santa Cruz Biotechnology) were used as controls. Semiquantitative measurement of ERα protein expression per cell was achieved using a Vickers M85 scanning and integrating microdensitometer (Vickers, York, UK),[24,25] operating at a wavelength of 550 nm; the objective was ×40; mask A3. At least 20 readings were made in triplicate sections of each tibia ($n = 5$ rats in OVX, sham-operated, control, and loaded groups). The amount of ERα protein content per osteocyte was measured in four distinct regions of the proximal tibias from sham and OVX rats (Fig. 1) and in seven regions of transverse sections from control and loaded rat ulna (Fig. 2A). The position of the rat ulnar neutral axis for the rat ulna had been calculated previously.[19] The distance of each region from the neutral axis was used to calculate the peak strain magnitude during locomotion and artificial loading. Results are presented as the mean integrated extinction × 100 (MIE × 100) per cell ± SE. Whereas the limitations of immunolabeling prevented a fully quantitative, stoichiometric assessment of ERα protein expression per cell, this analysis provides a semiquantitative assessment of expression levels.

Effect of mechanical strain and estrogen on ERα mRNA expression in vitro

ROS 17/2.8 cells were seeded at a density of 80,000/strip and 275,000/dish (60 mm) and maintained in DMEM containing 10% FCS. After washing twice in PBS, cells were incubated overnight in DMEM containing 2% FCS (charcoal/dextran-stripped). Cells in dishes were exposed to estrogen (10^{-8} M) and strain was applied by loading the strips

TABLE 1. REAL-TIME PCR PRIMER SEQUENCES (5′ → 3′)

Primer	*Sequence (5′ → 3′)*	*Position and length*	*Accession no.*
ERα forward	CCAATTCTGACAATCGACGC	679–918	NM_012689
ERα reverse	TCTTATCGATGGTGCATTGGTT	239 bp	—
β-actin forward	CTATGAGCTGCCTGACGGTC	912–798	BC063166
β-actin reverse	AGTTTCATGGATGCCACAGG	104 bp	—

onto which cells had been seeded in four-point bending in a custom-designed loading apparatus (1 Hz, 3400 με, 600 cycles[9]). Control and treated cells on strips and dishes were maintained under similar conditions for specified times after treatment. Total RNA was extracted from control and treated cells at specified time-points using Trizol Reagent (Invitrogen, Paisley, UK) and treated with RNase-free DNase (Qiagen). Integrity of RNA was verified electrophoretically by ethidium bromide staining and by OD_{260}/OD_{280} nm absorption ratio > 1.95. Total RNA (0.5 μg) was reverse transcribed with 200 U of SuperScript II RNase H^- Reverse Transcriptase (Invitrogen), using 50 ng random primers (Invitrogen). Real-time quantitative PCR was carried out using QuantiTect Syber Green PCR kit (Qiagen) and Opticon 2 lightcycler (MJ Research, Waltham, MA, USA). Primer sequences, the positions in the coding region, and the expected real-time RT-PCR products for ERα and β-actin are summarized in Table 1. A relative standard curve was constructed for both ERα and endogenous reference (β-actin) using serial dilutions of cDNA, and these standards were included in each run. Samples of unknown concentrations were quantified relative to these standard curves. ERα mRNA levels were normalized to the reference gene to account for cDNA loading differences. Standards were run in duplicates and samples in triplicates. Average values were used for subsequent statistical analysis. The PCR conditions used a 15-minute initial enzyme activation step followed by 40 cycles of 15 s at 94°C, 30 s at 52°C, and 60 s at 72°C. The final elongation step was 7 minutes at 72°C.

Assessment of localization of ERα by immunostaining and confocal microscopy

For morphological studies, ROS 17/2.8 and MLO-Y4 cells were cultured and treated with either mechanical strain or exogenous estrogen as described above. At various time-points after treatment, cells were fixed in ice-cold methanol for 5 minutes. The cells were permeablized in Triton buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium azide, in PBS at pH 7.0) at 4°C and immunostained for ERα (Santa Cruz) overnight. The secondary antibody was FITC-conjugated goat anti-rabbit IgG (Molecular Probes). Scanning laser confocal microscopy was performed on a Leica TCS NT system (Heidelberg, Germany).[26,27] Fluorescent images were collected in sequential 1-μm steps through the cells for fluorescein isothiocyanate at 488-nm emission wavelengths. Confocal micrographs shown are merged through-focus images for a stack of *xy* images.

Effect of mechanical strain and estrogen on ERα promoter activity

Cells were seeded at a density of 50,000 cells/strip or 30,000 cells/well (6-well plates), maintained in DMEM containing 10% FCS, and transiently transfected by 3-h incubation with Effectene (Qiagen). The plasmids were used at a concentration of 1 μg DNA per strip/well. Human ERα promoter constructs[28] were a gift from Dr Shin-ichi Hayashi (Saitama Cancer Center Research Institute). The AB region of the ERα promoter has been shown to be involved in the autoregulation of ERα expression.[29] Promoter F has previously been shown to be used by osteoblast cells,[30] and the 46-kDa truncated ERα isoform is derived from this promoter.[31] To normalize for transfection efficiency, cells were also transfected with a *Renilla* expression plasmid (Promega) at a concentration of 0.5 μg DNA/strip/well. Results are expressed as Firefly luciferase activity normalized against *Renilla* luciferase activity, which was measured in 20 μl of cell lysates in opaque 96-well plates using the Dual Luciferase Assay System from Promega. Samples were measured on Wallac Victor 1420 multilabel Counter using Wallac 1420 Workstation software version 2 (Wallac Oy, Turku, Finland).

Statistical analysis

For clarity, some data are represented as percentages, but all comparisons were made with raw data. All results are reported as means ± SE. Differences between locations/regions within each treatment group were assessed using one-way ANOVA followed by the posthoc (Bonferroni) multiple comparisons method. Two-way ANOVA was used when comparing responses from sham with OVX animals and from control with loaded animals. Spearman rank correlation was used to determine the relationship between strain and ERα. $p < 0.05$ was considered statistically significant.

RESULTS

Regional distribution of ERα expression in osteocytes and osteoblasts in the tibia

Immunolabeling with polyclonal anti-ERα antibodies showed that the majority of osteocytes and all periosteal cells adjacent to the bone surface (presumptive osteoblasts and lining cells) contained ERα predominantly in the cytoplasm (Fig. 1). The labeling intensity of osteoblasts and lining cells was much higher than osteocytes (Fig. 1Aj). Because of problems of identification and overlap of presumptive osteoblasts and lining cells, we confined our study

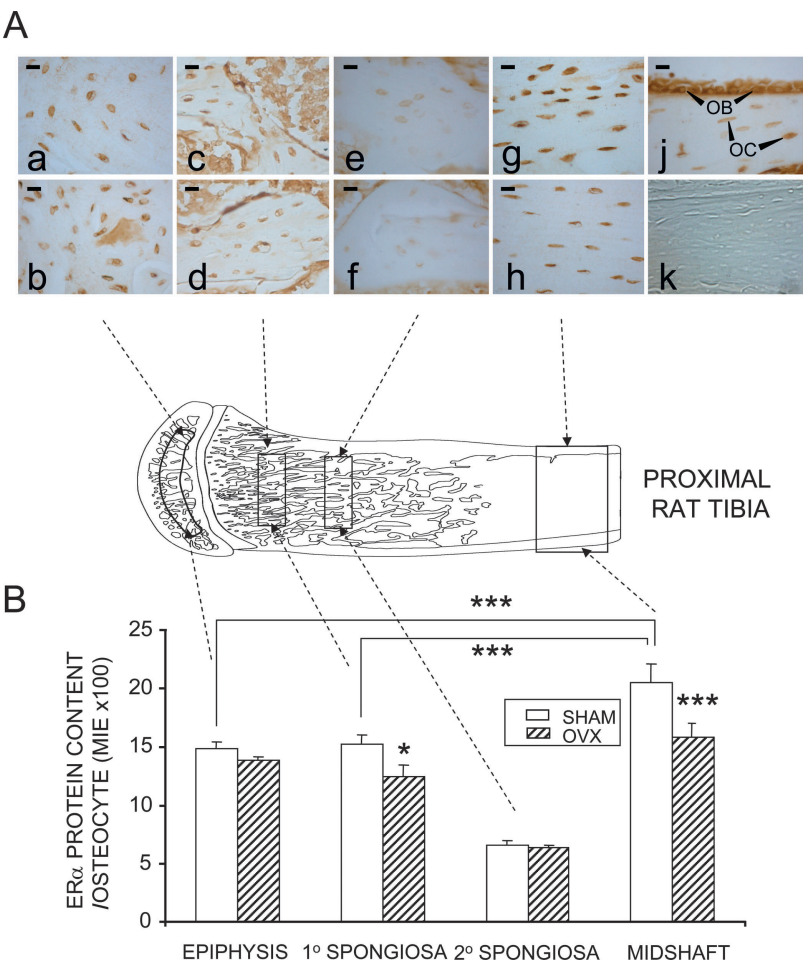

FIG. 1. (A)Immunolocalization of ERα in paraffin-embedded tibia sections, showing osteocytes in epiphysis (a and b), 1° spongiosa (c and d), 2° spongiosa (e and f), and midshaft (g and h) of sham (a, c, e, and g) and ovariectomized (b, d, f, and h) rats. Comparative staining in osteoblasts (OBs) and osteocytes (OCs) (g). Negative control section with phase contrast to show aspects of histology (k). (B) Midshaft osteocytes from the sham animals expressed significantly higher levels of ERα than the osteocytes of the trabecular bone. 2° spongiosa osteocytes from the sham animals had significantly lower levels of ERα than the osteocytes of the other trabecular regions measured. In ovariectomized animals, the ERα content per osteocyte was significantly less than in intact controls (sham operated) both in the cortical midshaft and the primary trabecular spongiosa. Data represent mean ± SE; $n = 5$. *$p < 0.05$; ***$p < 0.001$. Scale bar = 12 μm.

to osteocytes. These cells are highly responsive to changes in their strain environment.[32,33]

Previous evaluations of ERα expression in osteocytes relied on the assessment of the proportion of cells showing positive immunohistochemical labeling. Such evaluations are dependent on the immunochemical conditions used for detection and will depend largely on the arbitrary setting of the minimum positive level of labeling per cell.[34] This may account for the wide (14–65%[23,35–37]) variation in the reported proportion of ER-"positive" osteocytes. In this study, 92 ± 1.1% of osteocytes in the cortical bone of the midshaft expressed readily detectable levels of ERα (Fig. 1Ag). This contrasts with those within the secondary spongiosa (Fig. 1Ae) where levels were low enough to be scarcely distinguishable from background in control sections without primary antibody. Although assessment of the numbers of $ERα^+$ osteocytes depends predominantly on the arbitrary setting of a threshold, the relative values clearly show that the numbers of $ERα^+$ osteocytes in these trabecular regions are lower than in the cortex.

To provide a semiquantitative evaluation of the levels of ERα expression in osteocytes in various bone compartments, we used scanning and integrating microdensitometry. This provides an accurate measure of the level of ERα expression per osteocyte from the amount of reaction product per cell.

This approach disclosed regional differences. In the cortical bone of the midshaft, the osteocytes express significantly higher levels of ERα per cell (20.9 ± 0.6 [MIE × 100 ± SE]; $p < 0.001$) than those in the trabecular bone of the primary spongiosa (15.2 ± 0.5) and epiphysis (15.2 ± 0.4; Fig. 1B). Within these trabecular regions, there is a gradation of detectable osteocytic ERα protein content, with significantly lower levels per osteocyte in the secondary (6.6 ± 0.3; $p < 0.001$) than in the primary spongiosa (Fig. 1B).

Effects of OVX on ERα expression in osteocytes

To determine whether estrogen levels affect ER expression in osteocytes, we compared the ER content per cell in tibias of OVX and sham-operated rats. This showed that the ERα content per osteocyte in OVX animals was significantly less than in (sham-operated) intact controls both in the cortical midshaft (–24%; $p < 0.001$) and the primary spongiosa (–19%; $p < 0.05$; Fig. 1B). The OVX-related fall in ERα expression in these regions of the bone contrast with the lack of change in the osteocytes of the secondary spongiosa. However, even in animals with intact ovaries, levels of ERα protein expression in the secondary spongiosa were sufficiently low to be close to background. The absence of any effect could therefore be caused by lack of detection.

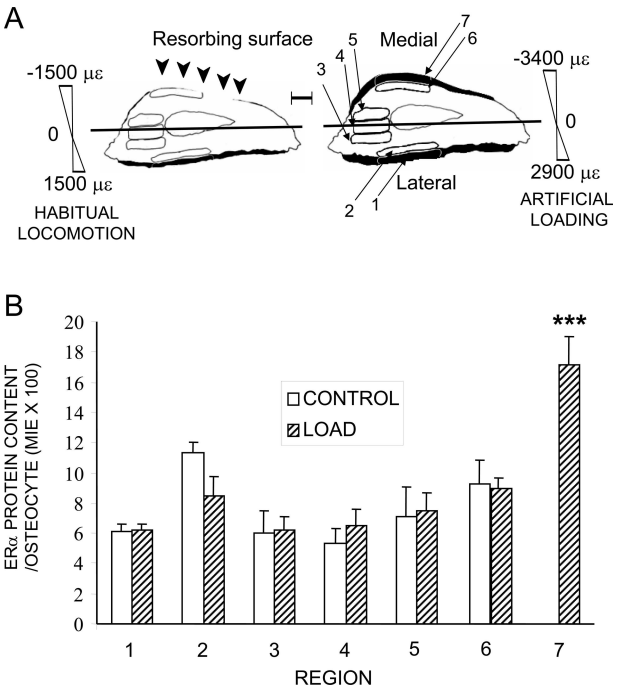

FIG. 2. (A) Images of the rat ulna at one cross-sectional level distal of the midshaft showing tensile and compressive strains. Arrowheads point to an area of periosteal resorption on the medial face of the nonloaded ulna. There is an increase in mineral apposition rate on the lateral face and a complete reversal of resorption to active formation on the medial face in response to mechanical loading. New bone formation is shown as filled regions (regions 1 and 7). (B) ERα protein content per osteocyte in different regions of rat ulna (regions 1–6 in habitual locomotion and regions 1–7 in artificially loaded) was measured by microdensitometry at one cross-sectional level distal of midshaft. Control animals were exposed to habitual strain and the treated group's ulna were axially loaded (3400 με). Although there is no regional comparator for region 7, expression of ERα protein per osteocyte in region 7 was significantly higher than all other regions of the cross-section (regions 1–6). Data represent mean ± SE; $n = 5$. ***$p < 0.001$. Scale bar = 1 mm.

Effects of mechanical strain on expression of ERα in osteocytes

To determine whether OVX-induced downregulation of ERα is compensated for by upregulation caused by increased strain, we examined whether short, potentially osteogenic, periods of dynamic loading also regulate ERα expression. The ulna loading technique[18,19] involves superimposing short periods of dynamic loading 3 days a week for 2 weeks onto the loading regimen engendered by the animals' own habitual locomotion. This extra loading stimulated an increase in bone formation rate on the bone's lateral (longitudinal tension) periosteal surface and transformed the growth-related modeling activity on the medial (longitudinal compression) surface from resorption to formation (Fig. 2A).

Analysis of the ERα expression per osteocyte in the cortical bone of the distal diaphysis revealed a significant influence of location within the bones cross-section ($p = 0.007$; Fig. 2B). Spearman rank correlation between strain level and ERα content per osteocyte showed that, in both control and artificially loaded limbs, there was a low correlation ($r = 0.26$ and 0.30, respectively) between an osteocyte's predicted peak locomotor or artificial strain and the amount of ERα protein per osteocyte. Because the strain distribution is a product of the bone's shape, it is impossible to distinguish the effects of strain and location. The only conclusion to be drawn from these data, therefore, is that, within a single cross-section at the midshaft, there is little difference in ERα content per osteocyte related to osteocyte age (depth within the cortex), relationship to periosteal or endosteal surface, or habitual strain level. The only exception to this is the substantially higher level of ERα content per osteocyte in the new bone deposited on the medial periosteal surface in artificially loaded bones. Expression levels at this region exceeded those evident in all other regions including bone laid down at a similar time on the lateral surface. Because the medial surface is undergoing resorption in control bones, there is no direct regional comparator. Nevertheless, the expression of ERα protein per osteocyte in this region (region 7) in these artificially loaded bones was significantly higher than in all other regions of the cross-section[1–6] (Fig. 2B; $p < 0.001$).

Effect of mechanical strain and estrogen on ERα mRNA expression

To establish a direct association between bone cells' stimulation by estrogen or mechanical stimulation, we studied mRNA levels of ERα using real-time RT-PCR in ROS 17/2.8 cells. Specificity of the PCR products was confirmed by high-resolution gel electrophoresis (Fig. 3A). Melting point analysis showed a single peak at 82.2°C for ERα and 81.8°C for β-actin. Both strain and estrogen induced short-term changes in ERα mRNA expression (Fig. 3B), suggesting use, degradation, and formation.

Changes in mRNA levels of ERα associated with estrogen followed the same time-course as those following strain but were somewhat later and smaller. Strain caused a significant reduction (75%; $p < 0.01$) in ERα mRNA levels 6 h after treatment. The reduction associated with estrogen was much smaller (34%; $p < 0.01$) and became significant only after 24 h. By 24 h after strain, the strained cells had already recovered from ERα downregulation, and there was a significant increase (60%; $p < 0.01$) of ERα mRNA expression levels above those seen in nonstrained cells.

Thirty-two hours after strain or estrogen, the ERα mRNA levels reverted back to their control values.

ERα promoter activity in human osteoblast-like cells

To assess ER gene transcription, we studied differential ER promoter use in human osteoblast-like (HOS) cells basally and in response to estrogen and strain. Three different ERα promoter constructs (of seven promoters currently recognized[31]) linked to luciferase activity, and representing promoter regions AB, F, and C,[28] were used to determine ERα promoter activation in response to estrogen or strain in HOS cells. The cells were transfected 24 h before treatment and harvested 24 h later. Treatment with estro-

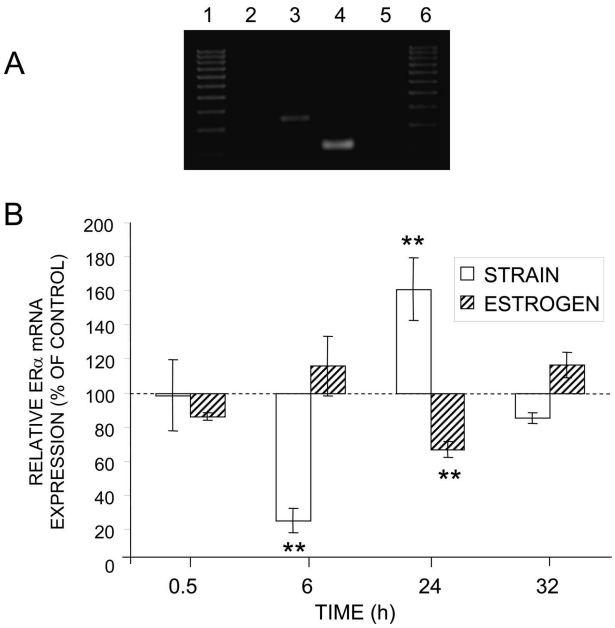

FIG. 3. (A) Agarose gel (2.5%) electrophoresis of real-time RT-PCR products derived from ROS 17/2.8 cell total RNA. Lanes 1 and 6: 100-bp DNA size marker; lane 3: ERα; lane 4: β-actin. Effect of mechanical strain (3400 με) and estrogen (10^{-8} M) on ERα mRNA expression levels in ROS 17/2.8 cells. (B) ERα mRNA levels were measured using real-time PCR and normalized against β-actin mRNA levels. Both mechanical strain and estrogen resulted in a significant decrease in ERα mRNA levels. After the initial reduction, there was a marked increase in ERα mRNA levels in response to mechanical strain. Both strain- and estrogen-induced changes in ERα mRNA levels reverted back to control levels at 32 h after treatment. Data represent mean ± SE; ($n = 5$). $^{**}p < 0.01$.

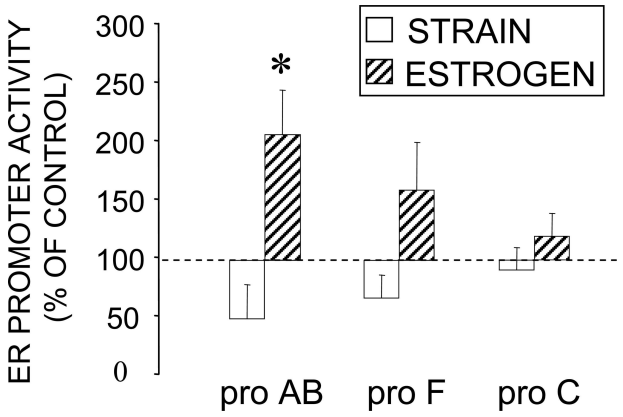

FIG. 4. Percentage changes in human ERα promoter activity compared with respective controls. HOS cells were transfected then exposed to a 10-minute period of mechanical strain or 10^{-8} M 17β-estradiol and harvested 24 h later. 17β-estradiol stimulated statistically significant increases in promoter AB ($a = 0.04$, $n = 6$). Mechanical strain did not stimulate any statistically significant changes in any of the promoter constructs AB ($n = 3$).

gen caused modest changes in the promoter activity of pro AB and pro F (Fig. 4), but these increases were statistically significant only for promoter AB (200%; $p = 0.04$; $n = 6$). Mechanical strain produced no significant changes in pro AB or pro F. Promoter C was previously shown to be active in endometrium-derived cells[28] but failed to show activation in bone cells in response to estrogen or strain. This highlights the possibility of tissue-specific promoter use in the control of ERα expression. Consequently, promoter C was used as a negative control in this study. The lack of promoter activity in response to mechanical strain is consistent with the observed reduction in ERα mRNA levels 6 h after strain. Increased promoter activity coupled to changes in mRNA stability may explain why the ERα mRNA levels drop more slowly after estrogen treatment. These findings are consistent with the in vivo data and support the conclusion that estrogen has substantial effects on ERα expression levels in bone cells but that strain does not.

Translocation of ERα in MLO-Y4 and ROS 17/2.8 cells

In cells of the osteocyte-like MLO-Y4 cell line, both estrogen (10^{-8} M) and a period of mechanical strain (3400 με, 1 Hz, 600 cycles) caused a marked translocation of ERα from the cytoplasm to the nucleus (Fig. 5B). Strain was associated with a similar rapid translocation of ERα to the nucleus (Fig. 5A). Retention of ERα within the nucleus was more prolonged in cells exposed to estrogen than those treated with strain. Twenty-four hours after either strain or estrogen, ERα distribution reverted back to that in untreated cells. A similar pattern of nuclear translocation was observed in osteoblast-like ROS 17/2.8 cells in response to both mechanical strain and estrogen (Fig. 5C, 1h-L and 1h-E). Strain but not estrogen also induced discrete membrane localization of ERα in osteoblast-like cells within 10 minutes of treatment (Fig. 5C, 10min-L). This suggests a nongenomic targeting of ERα in response to mechanical strain. Although there was an indication of strain-induced ERα localization at the cell membrane of the osteocyte-like cells (data not shown), the staining was not as robust as that observed in the osteoblast-like cells.

DISCUSSION

Improved immunocytological technique has resulted in progressively higher estimates of the number of osteocytes containing ERα.[35–37] Our present data from rat tibias suggest that 92% of osteocytes express ERα. This agrees with other reports.[23,37] However, we also show clear regional differences in the expression levels of ERα per osteocyte. Expression was higher in the midshaft cortical bone than in any cancellous region and lowest within the secondary spongiosa. To our knowledge, only two previous reports compare ERα expression in different compartments of bone. Our findings agree with that reporting ERα expression at the protein level[23] and contrast with that reporting that ERα mRNA message was observed only in cancellous bone.[38]

In agreement with earlier reports,[35,38] we showed that OVX is associated with decreased levels of ERα expression per osteocyte. The largest decrease occurs in the cortical midshaft of the tibia, where ERα is normally most abun-

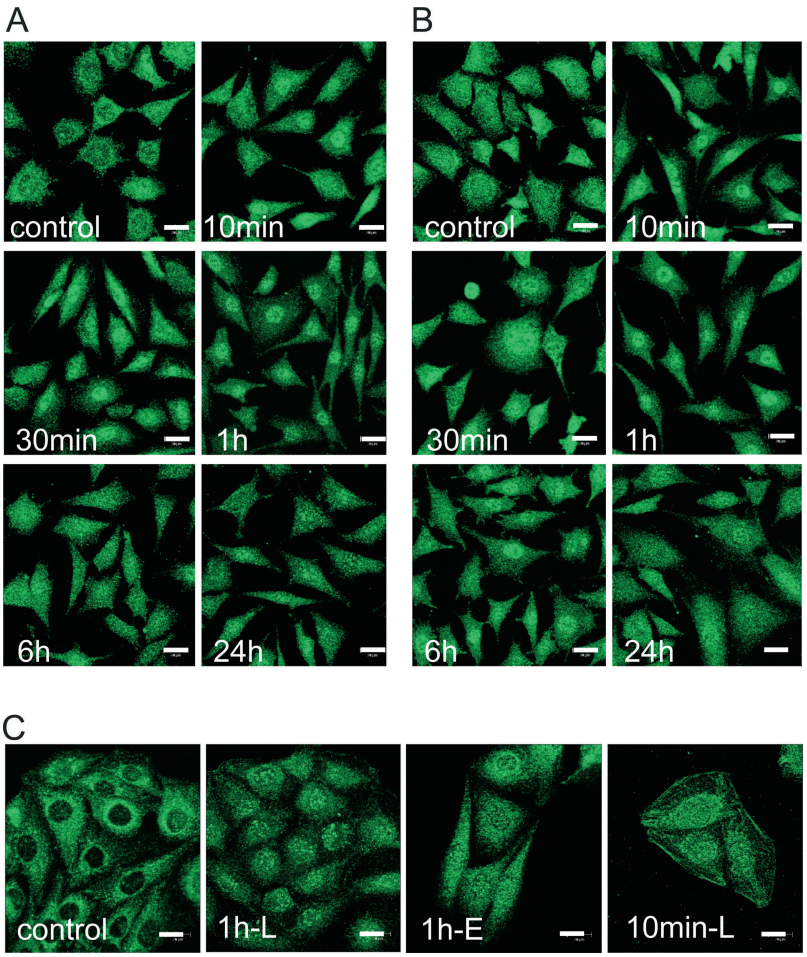

FIG. 5. ERα showed a predominantly cytosolic distribution in untreated cells. Both (A) mechanical strain and (B) exogenous estrogen caused a marked translocation of ERα to the nuclei within 10 minutes of treatment, which persisted in estrogen-treated cells 6 h after treatment, whereas this effect was short lived in mechanical strain–treated cells. After 24 h of either treatment, ERα distribution reverted back to that observed in untreated cells. (C) A similar pattern of nuclear translocation was observed in ROS 17/2.8 cells in response to both mechanical strain (1 h after treatment, 1h-L) and exogenous estrogen (1 h after treatment, 1h-E). Interestingly, there was also a discrete membrane localization of ERα within 10 minutes of application of mechanical strain (10min-L). Scale bar = 20 mm.

dant. The smallest decrease occurs in the cancellous secondary spongiosa, where its abundance is least. In contrast with this apparent sensitivity to estrogen status and inconsistent with our previous report,[36] we could only show a small, if significant, correlation between an osteocytes' strain history and its ERα content. This could not be separated from the potential relationship between ERα content per cell and location. In loaded bones, the only osteocytes showing substantially elevated levels of ERα were those in recently formed bone that had been stimulated by loading to be deposited on a site of reversal from previous resorption. The reasons for such high levels of ERα per osteocyte in this region are not clear. This bone was deposited during the same 2-week period as that on the lateral surface where ERα protein levels per cell were much lower. Its peak strain during artificial loading would have been higher than that of the similarly aged new bone on the medial surface but only marginally higher than that of the older bone onto which it was deposited. The unique feature of this new bone, distinguishing it from all other regions, is the strain-related reversal of the surface resorption of normal growth-induced drift to formation. It is tempting to speculate that change in osteocyte ERα expression plays a significant role in this process.

At the ERα mRNA and promoter levels, both mechanical strain and estrogen induced short-term changes in bone cells that reverted to control values within 32 h. This transience of effect is consistent with the findings in vivo showing that short periods of mechanical load, sufficient to engender an osteogenic response, have no lasting effect on ERα mRNA expression. Data of differential ER promoter use were consistent with findings in vivo showing that mechanical strain had no effect on transcriptional activity of the ER promoter domains tested. This suggests that the small changes in mRNA levels observed in response to mechanical strain are caused by modulation of ERα mRNA stability rather than transcriptional regulation.[39] However, we cannot exclude the possibility that ERα promoter regions, other than those studied here, are involved. Estrogen, on the other hand, significantly enhanced transcription through ERα promoter use, and by this means, may contribute to regulation of ERα expression in osteocytes in vivo.

In the traditional model for estrogen action, the hormone binds to its receptor in the cytoplasm of responsive cells, and the complex of ligand-bound dimerized receptor translocates to the nucleus where it binds to estrogen response elements of the DNA. In vitro, we found that in untreated osteocytes and osteoblast-like cells, ERα was mainly cytosolic. This contrasts with some earlier reports[23,40,41] sug-

gesting that ER resides predominantly in the nucleus but is consistent with other reports that it is primarily located within the cytoplasm.[42–45] Some of this disagreement may relate to cell or tissue type.

Our data agree with reports[43,46,47] that estrogen causes ERα to translocate to the nucleus. However, we are not aware of any previous report that, in osteoblast-like cells, strain induces ERα migration both to the nucleus and to the cell membrane. Some of the responses to estrogen are too rapid to be accounted for by changes in gene expression.[48–50] Nongenomic actions of estrogen can be ER-independent,[51–53] ER-dependent,[54–56] or a mixture of the two[57–59] mediated through growth factor receptor tyrosine kinases, such as the IGF-I receptor.

Our experiments give no indication of the mechanism of OVX-induced bone loss. However, there is clear evidence that, in the presence of ERs, both mechanical strain and estrogen protect bone against bone loss by reducing the activation frequency of negatively balanced remodeling units. The cortical bone from intact animals has all three factors (ER, strain, and estrogen) present. In the rat tibia, even after OVX, ERα content per osteocyte in cortical bone is at least as high as that in the epiphysis and the primary spongiosa and significantly higher than that in the secondary spongiosa of intact animals. Therefore, even after OVX, strain can mediate its antiresorptive effects in the bone cortex through relatively high levels of ERα. This contrasts with the situation in the spongiosa where lower levels of strain will be compounded by the loss of circulating estrogen levels and consequent reduction of estrogen receptors. This may account for the higher levels of bone loss observed in this compartment.

We have hypothesized[3] that, because full expression of bone cell's early osteogenic response to mechanical strain involves ERα, that the bone loss that accompanies estrogen withdrawal at menopause may be a consequence of less effective responsiveness to strain caused by downregulation of ER,[4,8,9,60] resulting from estrogen deficiency. It is easier to find support for low responsiveness to exercise in the absence of estrogen[61–66] than it is to link it with levels of ER. There is increasing evidence that ERα is involved in many other reactions that do not involve estrogen,[67–70] and so there is no conceptual reason why ERα regulation should be the sole prerogative of the receptor's primary ligand. The regional variation in osteocyte ERα concentration that we report is more likely to be the result of some local regulation than exposure to differing amounts of estrogen. One such local regulator could be the mechanical environment. However, within the limitations of the experiments reported here, we could see no evidence of substantial regulation of ERα in osteocytes in response to natural or artificially engendered strain within their surrounding bone.

In conclusion, almost all osteocytes in the rat tibia express ERα. Osteocytes in the cortical bone of the midshaft express significantly higher levels per cell than those in the trabeculae of the metaphysis and epiphysis. Within these regions, there is a gradation of ERα protein per cell with the osteocytes of the epiphysis and primary spongiosa having higher concentrations than the secondary spongiosa.

OVX is associated with a decrease of ERα per osteocyte in the cortex, where levels are initially high, and has no effect in the secondary spongiosa where initial levels are low. Strain history has only a small but positive correlation with the level of ERα per osteocyte. Osteocytes in bone stimulated by loading to be deposited on a previously resorptive surface have significantly higher ERα expression than those in, similarly aged, new bone deposited on a previously forming surface.

In osteocytes and osteoblasts in situ and in osteocyte and osteoblast-like cell lines in vitro, ERα is predominantly located in the cytosol. Strain as well as estrogen stimulates transient translocation of ERα to the nucleus. Strain but not estrogen also induces discrete membrane localization of ERα.

In bone cells in vitro, both estrogen and strain engender transient changes in ERα mRNA levels. The changes associated with strain are larger and earlier than those associated with estrogen. Estrogen, but not strain, upregulates ERα promoter activity.

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