Estrogen has rapid tissue-specific effects on rat bone


Estrogen has rapid tissue-specific effects on bone growth, wirelessly (37). In growing rats, ovariectomy (OVX) results in accelerated longitudinal (30) and radial (39) bone growth, as well as cancellous osteopenia (47), changes that are prevented by treatment with estrogen (39, 46). The skeletal changes in mature OVX rats are similar to the changes in postmenopausal women and consist of site-specific cortical and cancellous bone loss (10, 16). The former bone loss is due to a net increase in endocortical bone resorption; no bone is lost from the periosteal surface (39). The latter bone loss is associated with an increase in bone remodeling; there are increases in bone formation as well as in bone resorption (10, 29, 45).

Estrogen treatment prevents cancellous osteopenia in mature OVX rats by reducing the overall rate of bone remodeling, as well as by equalizing the magnitude of bone formation and bone resorption during the bone remodeling cycle (44, 46). Estrogen is essential in maintaining bone mass in the adult female skeleton, while at the same time the hormone inhibits bone formation when administered to estrogen-deficient laboratory animals and humans. These paradoxical effects of estrogen on bone metabolism can be reconciled by hypothesizing that the primary effect of the hormone is to prevent initiation of new bone remodeling units.

The initial step in the bone remodeling cycle is activation of focal bone resorption. Normally, most of the bone that is locally lost during this resorption phase is restored during the subsequent formation phase of the remodeling cycle (7, 28). It follows that a reduction in the initiation of new bone remodeling units would immediately result in a reduction in total bone resorption and later, as the formation phase of each ongoing remodeling site was completed, lead to a coupled decrease in the overall rate of bone formation. Indeed, some investigators have proposed that the initial effect of estrogen on osteoblasts is a stimulation of bone formation (5) and that the long-term inhibitory effects of the hormone on bone formation occur entirely secondarily to the decrease in initiation of new sites of bone remodeling.

The purpose of the present study in the rat proximal tibial metaphysis, a cancellous bone site that is responsive to estrogen, was to identify estrogen receptor (ER) mRNA and to determine the earliest effects of estrogen on mRNA levels for extracellular matrix proteins and on signaling peptides (cytokines and growth factors) related to bone formation and resorption. Additionally, we evaluated the earliest effects of estrogen on bone matrix synthesis at multiple cortical and cancellous sites by measuring the incorporation of \(^{3}H\)proline into bone. This latter study was performed to test the hypothesis that estrogen treatment results in a transient increase in bone formation. Finally, we compared mRNA levels for identical immediate-response genes, genes for matrix proteins, and genes for signaling peptides in bone with those in the uterus. Our purpose was to determine the similarity of the estrogen-initiated time course and target tissue specificity of changes. By focusing on the early responses to estrogen, it should be possible to distinguish immediate actions of the hormone on osteoblast metabolism from long-term changes that occur secondarily to decreased bone resorption.

METHODS

mRNA isolation and analysis. Recently (~1 wk) OVX 3-mo-old Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing ~250 g, were studied; estrogen was previously reported to stimulate bone formation in rats of similar age (5). The rats were euthanized in groups of three to...
four at 0, 1, 2, 4, 8, 16, 24, and 32 h after the subcutaneous injection of a 0.1-ml solution containing 500 µg/kg diethylstilbestrol (DES; Sigma Chemical; St. Louis, MO) dissolved in 50% ethanol. Time course and dose-response studies have revealed that this dose of ethanol has no effect on mRNA levels for any of the genes assayed in these studies (40). All groups were euthanized between 0900 and 1500 to minimize possible diurnal variation in message levels. DES was employed because it is a complete estrogen agonist and does not interact with, and is not metabolized to, other classes of steroid receptors. In a separate experiment to evaluate the effects of estrogen on immediate-response genes, OVX rats to be killed after 1 h were injected (n = 3–5) with the estrogen-receptor antagonist ICI 182,780 (Zeneca Pharmaceutical, UK) or vehicle. ICI 182,780 was added to the solution to reduce the possibility of interference from endogenous estrogen. Euthanasia was achieved by 10.220.33.4 on April 2, 2017 http://jap.physiology.org/ Downloaded from

#### Table 1. Assayed mRNAs

<table>
<thead>
<tr>
<th>Probe</th>
<th>Assay</th>
<th>Source of Probe</th>
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<tbody>
<tr>
<td>c-fos</td>
<td>Northern</td>
<td>Salk Institute (San Diego, CA; I. Verma)</td>
</tr>
<tr>
<td>c-jun</td>
<td>Northern</td>
<td>University of California (Berkeley, CA; R. Turner)</td>
</tr>
<tr>
<td>Osteocalcin</td>
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<td>Genetics Institute (Cambridge, MA)</td>
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<tr>
<td>Type I collagen</td>
<td>Northern</td>
<td>University of Connecticut (Farmington, CT; A. Heydermann)</td>
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<tr>
<td>TGF-β1</td>
<td>Northern</td>
<td>National Institutes of Health (Bethesda, MD; M.I. Sporn)</td>
</tr>
<tr>
<td>18S</td>
<td>Northern</td>
<td>Lofstrand Labs (Gaithersburg, MD)</td>
</tr>
<tr>
<td>ER-α</td>
<td>RT-PCR</td>
<td>Mayo Clinic (Rochester, MN; A. Maran)</td>
</tr>
<tr>
<td>ER-β</td>
<td>RT-PCR</td>
<td>Mayo Clinic (A. Maran)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>RNase Protection; RPA II</td>
<td>Mayo Clinic (A. Maran)</td>
</tr>
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<td>TGF-β1</td>
<td>RNase Protection; mCK-3</td>
<td>Pharmingen (San Diego, CA)</td>
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<td>TGF-β2</td>
<td>RNase Protection; mCK-3</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>RNase Protection; mCK-3b</td>
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<td>IFN-γ</td>
<td>RNase Protection; mCK-2</td>
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<td>IFN-β</td>
<td>RNase Protection; mCK-2</td>
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</tr>
<tr>
<td>LT-β</td>
<td>RNase Protection; mCK-3b</td>
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</tr>
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<td>GAPDH</td>
<td>RNase Protection; mCK-2 and mCK-3</td>
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</tr>
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<td>MIF</td>
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<td>L32</td>
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<td>IL-6α</td>
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<tr>
<td>IL-10</td>
<td>RNase Protection; mCK-2</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>TGF-β1, β2, and β3; transforming growth factor-β1, -β2, and -β3, respectively; ER-α and -β, estrogen receptor-α and -β, respectively; IGF-1, insulin-like growth factor 1; IFN-γ and -β, interferon-γ and -β, respectively; LT-β, lymphotoxin-β; glyceraldehyde-3-phosphate dehydrogenase; IL-1α, IL-1β, IL-6, IL-10, and IL-12; interleukin-1α, -1-receptor agonist, -6, -10, and -12, respectively; MIF, migration inhibition factor; TNF-α, tumor necrosis factor-α; L32, ribosomal protein; mCK, mouse cytokine chemokine template sets.</td>
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Before Northern analysis, RNAs were separated electrophoretically on a 1% agarose gel, transferred to an Amersham Hybond nylon membrane (Arlington Heights, IL) overnight via capillary action, and vacuum-dried at 80°C for 2 h (Precision Vacuum Oven; Chicago, IL). The membranes were then prehybridized for 2–4 h at 65°C in a buffer containing 50% deionized formamide, 10% dextran sulfate, 5× standard saline citrate solution buffer, 2× Denhardt’s solution, and 100 µg/ml sonicated, heat-denatured, single-strand salmon sperm DNA. Membranes were then hybridized for 80 min in the described buffer with the addition of 32P-labeled cDNAs for the genes tabulated in Table 1. Northern analysis was performed with probes for the protooncogenes c-fos and c-jun (at the 1-h euthanization), osteocalcin (OC), type I collagen, transforming growth factor-β1 (TGF-β1), and 18S RNA. mRNA levels were then normalized against 18S rRNA to control for unequal RNA loading.

Insulin-like growth factor I (IGF-I) mRNA was assessed by a RNase protection assay (RPA) employing the RPA II kit (Ambion, Austin, TX), which has been described (40). TGF-β1, TGF-β2, and tumor necrosis factor-α (TNF-α) mRNAs were measured by RNase protection with the mouse cytokine (mCK)-3 kit, whereas interferon-γ (IFN-γ), IFN-β, lymphotoxin-β (LT-β), interleukin-1β (IL-1β), IL-1-receptor antagonist (IL-1Ra), IL-6, IL-10, IL-12, and macrophage-migration inhibition factor (MIF) were assessed by the mCK-2 kit (Pharmingen, San Diego, CA). TGF-β3 was assayed by the mCK-3b kit (Pharmagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L32 were included in probe sets of both the mCK-2 and -3 kits. The RPA and mCK kits both employ the same fundamental protocol. The probe (set) was hybridized to the target RNA(s) in excess, and free probe was digested with RNases. The remaining (hybridized/RNase-protected) probe was purified, sorted by size on a denaturing polyacrylamide gel, and autoradio-
graphed. The quantity of each RNA species was based on the signal intensities of the resulting bands. These were then normalized against 18S, GAPDH, or L32 to control for uneven gel loading. Densitometric values were determined by a phosphor-imager (Molecular Dynamics, Sunnyvale, CA) and analyzed by ImageQuant PC-based software (Molecular Dynamics).

RT-PCR for ER-α and ER-β. In a separate experiment, frozen bone (proximal tibial metaphysis) and spleen tissue samples from 3-mo-old OVX and ovari-intact rats (n = 5) were homogenized, and the total RNA was isolated by using RNXazol (Tel-Test, Friendswood, TX). Spleen was used as a negative control (41). RNA was treated with RNase-free DNase, extracted with phenol chloroform, and precipitated with ethanol. The mRNA was used as template for cDNA synthesis by RT using oligo(dT) primer. The cDNA was then diluted serially with 1× PCR buffer (Perkin-Elmer, Norwalk, CT) at a final concentration of 1:4. The cDNA at each dilution was amplified with the use of primers to coding sense sequences, nucleotide 70–89 (5′-AAGTCTGGGACGCAGTCCGTACGAT-3′), and antisense sequences, nucleotide 414–446 (5′-GCAGGACTGATAATGTCGCTACG-3′), of ER-β or to sense sequences, nucleotide 930–954 (5′-GGATTTGGCCAGCCCTGGCTGGCAGGC-3′) and antisense sequences, nucleotide 1225–1249 (5′-ATTGAGCTGCTGCTGGCTGGC-3′), of ER-α or to sense sequences, nucleotide 506–525 (5′-TCCCTAACAGGTGCACAGC-3′), and antisense sequences, nucleotide 795–814 (5′-AGATCCACACGGATACCT-3′), of GAPDH. Amplifications were performed by extending the PCR to 40 cycles. For amplifying ER-β cDNA, the denaturation was at 94°C for 1 min, annealing was at 56°C for 1 min, and elongation was at 72°C for 2 min. For amplifying ER-α cDNA and GAPDH cDNA, the denaturation was at 94°C for 1 min, annealing was at 51°C for 1 min, and the elongation was at 72°C for 2 min. The sizes of the ER-α and ER-β and GAPDH PCR products are 320, 367, and 309 base pairs, respectively. The PCR products were analyzed by agarose gel (1.5%) with 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)) followed by ethidium bromide staining. The sizes of the PCR products were confirmed with appropriate DNA molecular weight markers.

[3H]proline incorporation. To determine the effects of a more prolonged treatment with estrogen, recently OVX Sprague-Dawley rats (1 wk after surgery) of a similar age and weight as in the previous studies were injected with 500 µg/kg DES (sc) or carrier and euthanized at 1, 2, 3, or 7 days. A baseline group of animals was euthanized on day 0. All animals (n = 5/group) were radiolabeled with 50 µCi/rat in 0.05 ml (50% ethanol) [3H]proline (1×4.5×H-proline; 181 TBq/mmol; Amersham Life Sciences) 6 h before euthanization. Calvariae, femora, and humeri were excised, and the epiphysis was separated from the long bones by blunt dissection. The growth plates were shaved off femora and humeri, and the cancellous bone and marrow of the metaphysis were separated from the cortex with a periosteal lifter. The separated cortical and cancellous bone samples were then defatted overnight in 100% ethanol, dried at 100°C, weighed, and separately incinerated in a sample oxidizer (model B0306, Pakard Tricarb, Downers Grove, IL). Radiolabel incorporation was determined by scintillation counting (Beckman LS6800, Fullerton, MA). Disintegra-tions per minute per milligram dry weight were then calculated, and the data were expressed as a percentage of the time-matched control values.

Data analysis and statistics. The densitometric values were averaged for each mRNA, and treatment, time point, and SE were calculated. In the [3H]proline incorporation experiment, radioactivities were calculated as a percentage of control and averaged by bone and treatment group. These data were analyzed by statistical software (SuperANOVA; Abacus Concepts, Berkeley CA) with one-way analysis of variance, linear regression analysis, and Fisher’s protected least significant difference post hoc test. Significance was accepted at P < 0.05.

RESULTS

A representative RT-PCR experiment is shown in Fig. 1 for OVX rats. ER-α and ER-β mRNA were expressed in bone but not in spleen, whereas the mRNA for GAPDH was expressed in both organs. Dilution of GAPDH cDNA resulted in the expected decrease in signal intensity. Dilution analyses of the cDNAs for ER-α and ER-β indicate that ER-α is the predominant form of ER mRNA expressed in the proximal tibial metaphysis, being expressed at approximately four times the level of ER-β. Similar results were obtained for ovari-intact rats (data not shown).

High-quality total cellular RNA was obtained from metaphysis and uterus. A photograph of phosphorimages of representative RPAs (Fig. 2) is shown.

The effects of estrogen and ICI 182,780 on protooncogene expression are shown in Table 2. Steady-state mRNA levels for c-jun and c-fos were increased (2- to 4-fold) in bone and uterus 1 h after treatment with DES. No change in either gene, in either bone or uterus, was detected after treatment with ICI 182,780.

The time course effects of estrogen on signaling peptide expression in OVX rats are shown in Figs. 3–7. mRNA levels for IL-1α, IL-10, IFN-γ, TGF-β2, and LT-β were not detected in either bone or uterus. mRNAs for IGF-I, IL-6, TGF-β1, and TGF-β2 were detected in bone and uterus, although there were quantitative differences between the tissues; mRNA concentrations of TGF-β2 and TGF-β1 were higher in bone, whereas IGF-I and IL-6 were higher in uterus. IFN-γ mRNA was detected in bone but not uterus. In contrast, IL-1β, IL-12, MIF, and TNF-α mRNA were detected in uterus but not in bone. We were able to detect low levels of IL-1β and TNF-α mRNA in bone extracts when the amount of total cellular RNA assayed was increased to 20 µg. However, estrogen treatment had no effect on mRNA levels for these cytokines (data not shown).
DES resulted in a transient increase in uterine IGF-I mRNA levels, which became significant at 2 h and reached a peak value (1,960% of control) at 8 h (Fig. 3A). Steady-state mRNA levels for IGF-I decreased in bone after DES treatment compared with untreated rats (~50%) (Fig. 3B). DES treatment resulted in an increase in steady-state mRNA levels for IL-6 in uterus, which became significant at 2 h (Fig. 4) and achieved a peak value (200%) at 4 h. IL-6 message levels were not altered by DES treatment in bone. DES had no effect on TGF-31 (Fig. 5A) or TGF-32 (Fig. 5B) levels in uterus or bone. DES had no effect on IFN-3 message levels in uterus (data not shown). On the other hand, DES resulted in rapid (1–4 h after DES), brief (observed at no more than 2 consecutive time points), statistically significant two- to fourfold increases in uterine mRNA levels for IL-13, IL-13, MIF, and TNF-3x.

The effects of DES on mRNA levels for extracellular matrix proteins are shown in Figs. 6 (collagen) and 7 (OC). DES resulted in a transient increase in uterine IGF-I mRNA levels that increased in steady-state mRNA levels for collagen, which became significant at 4 h and reached a maximum value (500% control) at 32 h. The trend for a progressive increase in mRNA levels was statistically significant by linear regression analysis (r = 0.87; P < 0.0001). In contrast, mRNA levels for collagen in bone decreased with time, although the correlation coefficient for this tendency just failed to achieve significance (r = –0.33; P = 0.07). OC message was not detected in uteri from either control or estrogen-treated rats. mRNA levels for OC in bone gradually decreased after DES treatment. This tendency was statistically significant by linear regression analysis (r = –0.37; P = 0.04).

The time course effects of DES on [3H]proline incorporation into cortical and cancellous bone are shown in Fig. 8, A and B. DES resulted in a decrease in incorporation of the radiosotope, which became significant after 48 h of treatment at cortical bone sites (calvariae, femora, and humeri periosteum) and after 72 h at cancellous bone sites (femora and humeri).

**DISCUSSION**

The genomic effects of estrogen are believed to be mediated by specific receptors for estrogen (31). ERs have been identified in a rat osteosarcoma cell line (17), in primary cultures of human osteoblastic cells (6), in isolated avian osteoclasts (25), and in a cell line derived from a human giant cell tumor (28). In vivo, ER mRNA has been identified in periosteum (41), as well as in extracts from cancellous bone tissue (25), and ER mRNA and/or protein has been visualized in situ in cells of the osteoblast and osteoclast lineage in human, murine, and avian skeletal tissues (2, 11, 24, 34). Thus estrogen could have direct genomic as well as indirect effects on the cells that form and resorb bone.

Evidence for multiple receptors for estrogen was initially reported by Kon et al. (18), and two distinct receptors, ER-3 and ER-3, have now been conclusively demonstrated (19). The mRNAs for both ERs have been identified by RT-PCR in cultured bone cells, as well as in extracts from rat bone (25). In the present study, ER-3 was the predominant receptor expressed in bone. We have verified the expression of the mRNAs for the two receptors in the proximal tibial metaphysis, the skeletal site in which we investigated the effects of estrogen on gene expression for immediate-response, signaling peptide, and matrix protein genes. Our results differ from those of Onoe et al. (25), however, in that we consistently detected higher concentrations of ER-3 than ER-3.

Possible reasons for the discrepancy include differences in the tissues assayed (unlikely because, in unpublished studies, we obtained similar results for the metaphysis + periosteum); age (unlikely because, in unpublished studies, we have obtained similar results in 3-mo-old and 2-yr-old rats); gonadal status (unlikely because we have obtained similar results in...
OVX and ovary-intact rats); and primers used for the PCR. The primers may be important because a slight difference in the efficiency of the amplification could be responsible for the difference. For this reason, the most important conclusion from these results is that mRNA for both receptor isoforms was expressed in bone at the site where our measurements were performed. Determination of which receptor predominates in bone will require measurement of receptor number, a measurement that it is not yet feasible to perform.

A cascade mechanism has been proposed to explain the actions of estrogen on target cells (37). According to this model, estrogen binds to its receptor to form an active transcription factor that regulates the expression of a limited number of genes. The protein products of these immediate-response genes regulate the expression of signaling peptides that, in turn, regulate the expression of a large number of genes that mediate the overall change in bone metabolism. The initial evidence for sequential gene changes after estrogen treatment was obtained in reproductive tissues (37). The sequential changes in gene expression observed in this study provide in vivo support for this model in bone.

The mRNA levels for c-jun and c-fos were assayed because the expression of these immediate-response genes is thought to be crucial for the actions of estrogen on target cells. c-jun and c-fos are immediate early genes that are rapidly induced by various stimuli, including estrogen. The expression of these genes is believed to be important in the regulation of gene expression and cell proliferation.

Fig. 3. Steady-state mRNA levels for IGF-I normalized to 18S rRNA levels in uterus (A) and bone (B). Values are means ± SE (n = 3–4). Time interval, no. of hours after treatment with DES. DES resulted in increased message levels in uterus (A) and decreased message levels in bone (B). * P < 0.05.

Fig. 4. Steady-state mRNA levels for interleukin-6 (IL-6) mRNA expression normalized to L32 mRNA levels. Values are means ± SE (n = 3–4). Time interval, no. of hours after treatment with DES. DES increased message levels in uterus but not in bone. * P < 0.05.

Fig. 5. A: steady-state mRNA levels for transforming growth factor-β1 (TGF-β1) normalized to L32 mRNA levels. B: steady-state TGF-β2 mRNA expression normalized to L32 mRNA levels. Values are means ± SE (n = 3–4). Time interval, no. of hours after treatment with DES. DES had no significant effect on message levels in either uterus or bone.
genes are known to be regulated by estrogen in the uterus (20, 23) and because the protein products of the two genes form the AP-1 complex, a cellular transcription factor that regulates expression of many genes (9). Furthermore, gene knockout and overexpression studies in mice as well as cell culture studies suggest that c-jun and c-fos peptides are important physiological regulators of bone metabolism (21). The rapid upregulation of mRNA levels for c-fos and c-jun in tibia by DES but not by ICI 182,780 strongly supports ER-mediated regulation of these two genes in bone.

Steady-state mRNA levels for growth factors and cytokines that have been associated with estrogen-deficiency-induced increases in bone remodeling were assayed. These include factors that stimulate bone formation (IGF-I and TGF-β₁) and bone resorption (IL-1 and IL-6) (14, 22). We also measured additional factors that either have been implicated as promoters of bone resorption (TNF-α and IFN-γ) or have no known effect on bone (IL-12 and MIF) (22). These latter factors were evaluated to help establish the specificity of any observed changes in cytokine and growth factor mRNA levels.

IGF-I increases synthesis of bone matrix proteins in cultured bone cells (3) and bone growth in rats (13). The long-term effects of OVX include increases in IGF-I mRNA in bone (4) and systemic levels of the IGF-I peptide (15). Estrogen reduced IGF-I mRNA concentration in both cortical (33) and cancellous bone (1) in OVX rats. Similarly, serum IGF-I is reduced in estrogen-treated OVX rats (15). The reduction in IGF-I message within 2 h of estrogen treatment as well as the potent stimulatory effects of IGF-I on bone formation reported by most researchers suggest that decreased expression of this growth factor may be responsible for the rapid reduction in bone formation we observed in estrogen-treated OVX rats.

TGF-β₁ mRNA levels are positively correlated with bone formation in rat bone (4, 43). mRNA levels for TGF-β₁ are increased in the metaphysis shortly after
OVX (4) but are reduced in severely osteopenic bone after long-term estrogen deficiency (12, 43). These results suggest that the changes in TGF-β expression primarily reflect the reduction in osteoblast number that accompanies severe bone loss. This conclusion is supported by our failure to detect changes in mRNA levels for TGF-β1 by either RPA or Northern blot analysis after short-term estrogen treatment.

TGF-β3 has been implicated in mediating estrogen action on cultured bone cells (48). However, mRNA levels for TGF-β3 in the metaphysis were below the detection limit of the RPA in both control and estrogen-treated rats. Extraction of TGF-β protein from the metaphysis has revealed that TGF-β3 is a minor component of the TGF-β peptide that is deposited into bone matrix (8).

Short-term estrogen treatment had no effect on steady-state mRNA levels for cytokines that increase bone resorption, including IL-1, IL-6, TNF-α, and IFN-γ. In contrast, the hormone resulted in transient increases in mRNA levels for three of these cytokines in uterus; the exception was IFN-γ, which was not detected in that tissue. We cannot rule out the possibility that estrogen regulates peptide levels for these cytokines by posttranscriptional regulation. Additionally, it is possible that the hormone regulates the expression of one or more of these cytokines in a small subpopulation of cells in the metaphysis. Previous studies, however, have shown that mRNA levels for several of these cytokines become elevated in bone after high-dose ethanol (TNF-α) (40) and spaceflight (IL-1 and IFN-γ) (49), conditions that result in bone loss.

The mRNA levels for type 1 collagen and OC were assayed as indexes for estrogen-induced changes in extracellular matrix synthesis. The pronounced increase in steady-state mRNA levels for type 1 collagen in uterus was anticipated because of the pronounced growth-stimulating effect of the hormone on that organ. In contrast, the progressive decrease in mRNA levels for type 1 collagen and the osteoblast-specific protein OC in bone after estrogen treatment is not consistent with the direct anabolic effect of the hormone on osteoblasts proposed by some investigators (5). If estrogen had a direct stimulatory effect on osteoblast activity, we would have expected to observe an increase in mRNA levels for type 1 collagen after a time course similar to the uterine response. In contrast to estrogen, a single administration of parathyroid hormone increased steady-state mRNA levels for bone matrix proteins within 8 h (Turner, unpublished observations). The steady-state mRNA levels for these matrix proteins are closely correlated with bone formation (4, 43) and provide evidence for an estrogen-induced inhibition of osteoblast activity. This interpretation is supported by the subsequent decrease in incorporation of [3H]proline into bone matrix.

An estrogen-induced inhibition of [3H]proline incorporation was also detected in the periosteum of tibia, femur, and humerus. These findings are in agreement with the results of previous studies in cortical bone that evaluated mRNA levels for bone matrix proteins and dynamic bone histomorphometry (35, 42).

We have validated [3H]proline incorporation as an assay for bone matrix production in two animal models. Radioautography demonstrated that most of [3H]proline in rat long bones is localized in osteoblasts within 20 min and is localized in bone matrix within 6 h (42). Other studies in which the matrix was extracted and hydrolyzed, and [3H]hydroxyproline was separated from [3H]proline by using an amino acid analyzer demonstrated that changes in [3H]proline incorporation into quai bone matrix reflect collagen synthesis (38).

The estrogen-induced increases in mRNA levels for c-fos, c-jun, IGF-1, and type 1 collagen in uterus are well recognized (20, 23). Additionally, we demonstrated that estrogen also results in transient increases in uterine mRNA levels for IL-1β, IL-12, MIF, TNF-α, and IL-6. These findings suggest that estrogen activates signaling pathways in uterus that are not activated by the hormone in bone. Additionally, the observed tissue differences in the direction of the response of some of the genes (e.g., IGF-1 and type 1 collagen) to estrogen indicate that context is critically important to establishing the precise pattern of the estrogen-induced cascade in an estrogen target tissue.

Estrogen-induced inhibition of initiation of new bone remodeling units is the likely mechanism for the overall decrease in bone remodeling in estrogen-treated women and laboratory animals. There is, however, a lag period between the initiation of the bone resorption phase of the bone remodeling cycle and the coupled initiation of the bone formation phase of the cycle. As a consequence, suppression of initiation of bone remodeling would have no immediate impact on bone formation. The duration of the bone remodeling cycle in young rats has not been precisely determined. However, it is long, compared with the duration in the present study, which argues strongly against decreased bone remodeling as the likely mechanism for the observed rapid changes in osteoblast metabolism (32). Recent studies suggest that estrogen regulates the balance between bone formation and bone resorption during the bone remodeling cycle as well as the overall rate of bone remodeling (44). Interestingly, the cell-specific partial estrogen agonist domiphen reduces the overall rate of bone remodeling in adult OVX rats but increases trabecular thickness and restores cancellous bone volume in animals with established osteopenia (36). These findings suggest that it may be possible to dissociate the direct and indirect actions of estrogen on bone formation with tissue-selective partial estrogen agonists.

In summary, this investigation has shown that estrogen initiates sequential changes in mRNA expression for immediate-response, cytokine, and matrix protein genes in bone. These changes, while initially similar in bone and uterus, later showed tissue-specific differences. These findings demonstrate that estrogen does not act directly on cancellous bone at the proximal tibial metaphysis to stimulate bone formation. Instead, the progressive reduction in mRNA levels for bone matrix proteins and the later decrease in incorporation in
[\textsuperscript{3}H]proline into bone matrix indicate that estrogen's inhibitory actions on bone formation are rapid.

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