Expression of estrogen receptors and enzymes involved in sex steroid metabolism in the rat tibia during sexual maturation

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Abstract

Estrogens are essential for bone mass accrual but their role before sexual maturation has remained elusive. Using in situ hybridization and immunohistochemistry, we investigated the expression of both estrogen receptor (ER) α and β mRNA and protein as well as several mRNAs coding for enzymes involved in sex steroid metabolism (aromatase, type I and II 17β-hydroxysteroid dehydrogenase (17β-HSD), steroid sulfatase (STS) and type I 5α-reductase) on sections of tibial metaphyses before (1- and 4-week-old), during (7-week-old) and after (16-week-old) sexual maturation in female and male rats. ERα and ERβ mRNA and protein were detected in metaphyseal bone in lining cells, osteoblasts, osteoclasts and some osteocytes with no apparent differences in expression during development or between the sexes. In contrast, aromatase, type I and II 17β-HSD and type I 5α-reductase mRNAs were first detected in osteoblasts, osteoclasts and occasionally in osteocytes from sexual maturation (7-week-old rat) and onwards. Only STS was present before sexual maturation. To study the significance of ERα and β expression in bone before sexual maturation when circulating sex steroid levels are low, 26-day-old female and male rats underwent gonadectomy or 17β-estradiol (E2) supplementation (0·5 mg/21 days) during 3 weeks. Following gonadectomy, trabecular bone volume (TBV) was lower in males (P=0·03) and there was a trend towards reduction in females (P=0·057). E2 supplementation increased tibial TBV compared with controls in both genders as assessed by Mason–Goldner staining. These data suggest that the presence of ERs in bone cells before sex maturation might be of significance for bone mass accrual. Furthermore, based on the mRNA expression of the crucial enzymes aromatase and type I 17β-HSD, we suggest that bone cells in the tibial metaphysis acquire the intrinsic capacity to metabolize sex steroids from sexual maturation onwards. This process may contribute to the beneficial effects of estrogen on bone mass accrual, possibly by intracrinology.


Introduction

Recent clinical observations in three male patients, one with an inactivating mutation in the estrogen receptor (ER) α and two with an aromatase enzyme deficiency have clearly established that estrogen is pivotal for bone mass accrual during puberty and bone metabolism in males and females (Smith et al. 1994, Morishima et al. 1995, Carani et al. 1997). Still, androgens seem to fulfill an essential role in establishing sexual differences in the skeleton, in both rats and humans (Turner et al. 1994b, Dagogo-Jack et al. 1997, Vanderschueren et al. 1998). Evidence is gathering that androgens and estrogen can exert direct effects on bone cells, since both ERs (ERα and ERβ) and androgen receptor (AR) mRNA and protein have been demonstrated in osteoblasts, lining cells, osteocytes and osteoclasts in several species, including the rat, rabbit and human (Braidman et al. 1995, 2001, Kusec et al. 1998, Noble et al. 1999, Vidal et al. 1999, Windahl et al. 2000, van der Eerden et al. 2002b). However, to date ERα and ERβ expression in the metaphysis of rat tibiae before and during sexual maturation has not been extensively studied.

The sex steroids acting on bone cells may either be derived from the circulation or be metabolized locally in bone by a process called ‘intracrinology’ (Labrie et al. 2000). It has been established in a number of studies that intracrinology can occur in various target tissues of sex steroids such as breast and brain, at least in humans (Labrie et al. 2000). A number of key enzymes are involved in the
metabolism of the sulfated precursors dihydroepiandrosterone sulfate (DHEA-S) and estrone sulfate (E1-S), which are present in high amounts in the circulation, into the active metabolites testosterone and 17β-estradiol (E2). Steroid sulfatase (STS) catalyzes the formation of DHEA and E1 from the sulfated precursors, DHEA-S and E1-S respectively. Furthermore, type I 17β-hydroxysteroid dehydrogenase (17β-HSD) converts androstenedione into testosterone and E1 into E2, whereas type II catalyzes the conversion in the opposite direction. Aromatase mediates the conversion of the androgens, androstenedione and testosterone into the estrogens, E1 and E2. Finally, type I 5α-reductase irreversibly converts testosterone into dihydrotestosterone. In primary cultures of rat osteoblasts, rat and human osteoblastic cell lines and in spongosan obtained from patients undergoing orthopedic surgery, the mRNA expression and bioactivity of the enzymes aromatase, type I and II 17β-HSD, STS and 5α-reductase have all been demonstrated (Schweikert et al. 1995, Dong et al. 1998, Eyre et al. 1998, Saito & Yanaihara 1998, Jansen et al. 1999, Feix et al. 2001). Moreover, using in situ hybridization and immunohistochemistry, Sasano et al. (1997) have localized aromatase mRNA and protein and type I 17β-HSD immunoreactivity in lining cells and osteoblasts in sections of 16 human tibiae. This finding was confirmed for aromatase in a study by Oz et al. (2001). These data suggest that intracrinology might be an important source for bioactive sex steroids involved in bone metabolism. Until now, no reports have shown age-related expression of any of these enzymes in rat bone, particularly around sexual maturation.

Numerous studies have been performed to gain insight in the mechanism by which estrogens regulate bone mass. Many of these have focused on the postmenopausal bone loss in humans by using aged ovariectomized (OVX) rats or mice, when there is active bone remodeling (Wronska et al. 1986, Bagi et al. 1993). These models, however, are not suitable to study bone mass accrual during growth. Some studies have used younger animals, ranging between 6 weeks and 4 months, in which predominantly bone modeling takes place instead of remodeling. During this phase, high doses of estrogen have been described to increase trabecular bone volume (TBV) in 8-week-old OVX and 3-month-old intact female rats (both 4 mg/kg per day) (Tobias et al. 1991, Chow et al. 1992). These animals are, however, already sexually maturing or mature, at the start of the experiment. To date, little is known about the effects of estrogen withdrawal or supplementation before sexual maturation, when endogenous sex steroid levels are low (<35 pg/ml) or undetected (Dohler & Wuttke 1976, Saksena & Lau 1979) but bone mass accrual is high. Two studies have reported bone loss in mandibular condyles and in the temporomandibular joint after OVX in rats before or during early sexual maturation (Okuda et al. 1996, Yamashiro & Takano-Yamamoto 1998).

In this study we have focused on three topics. Using in situ hybridization and immunohistochemistry, we have surveyed the mRNA and protein expression of both ERs in the tibial metaphysis before, during and shortly after sexual maturation in female and male rats. Moreover, we studied the mRNA expression of various key enzymes involved in sex steroid biosynthesis in the tibial metaphysis at the same timepoints. Finally, we analyzed the effects of gonadectomy and E2 supplementation on TBV in the proximal metaphysis of tibiae from female and male rats starting just before sexual maturation when circulating levels of sex steroids are low.

**Material and methods**

**Animals**

Female and male Wistar rats were obtained from Harlan (Broeckman Instituut, Someren, The Netherlands). They were kept in a light- and temperature-controlled room (12 h light, 20–22 °C) with food and water freely available. Experiments were approved by the local ethical committee for animal experiments.

**Experiment 1**

The animals (n=4) were killed at 1, 4, 7 and 16 weeks of age by in vivo fixation (2% paraformaldehyde in 0.1 M phosphate buffer supplemented with 75 mM lysine monohydrochloride and 10 mM Na periodate) as described previously (van der Eerden et al. 2000). Tibiae were isolated and fixed in the same fixative for 24 h. Then the tibiae were decalcified in 15% EDTA, including 0.5% paraformaldehyde for 4 weeks. Tibiae were cut into halves in a sagittal orientation, processed for paraffin embedding and 5 µm sections were cut mid-sagitally.

**Experiment 2**

Twelve 26-day-old female and 12 male Wistar rats were ovariectomized by the dorsal approach under O2/N2/halothane anesthesia (n=5), orchidectomized (n=6) or sham-operated (females, n=7; males, n=6). Rats enter sexual maturation at around day 30, which lasts about 4 weeks (Tanner 1962). This means that at the start of the experiment rats were still sexually immature. Three weeks later, when the rats were halfway through sexual maturation, the animals were killed using a fatal dose of Nembutal (Sanofi Santé Animale, Maassluis, The Netherlands). Animals from each experimental group were fixed in vivo and tibiae were processed as mentioned above.

**Experiment 3**

Twelve 26-day-old female and 12 male Wistar rats (female average body weight: 63·2 g (56·4–68·8 g); male average body weight: 60·2 g (54·8–69·3 g)).
body weight: 78·8 g (69·2–90·8 g)) were implanted with a slow-release pellet (Innovative Research of America, Saratosa, FL, USA) s.c. between the scapulae, releasing 0·5 mg E2 (n = 6) over a 21 day period. The control groups received a placebo pellet (n = 6). After 3 weeks, the animals were killed using a fatal dose of Nembutal, fixed in vivo and their tibiae were processed as mentioned above.

Generation of cRNA probes for in situ hybridization

In situ hybridization probes for ERα and β have been described in detail in a previous study (van der Eerden et al. 2002b). The probes for rat aromatase, type I and II 17β-HSD, STS and type I 5α-reductase have all been described recently (van der Eerden et al. 2002a). Table 1 contains an overview of the cRNA probes, which were used in this study. Vector (10 µg) was linearized with the appropriate restriction enzymes. The probes were labeled with digoxigenin (DIG) (Roche, Basel, Switzerland) as described before (van der Eerden et al. 2002b) and hydrolyzed to reduce the probe size to 200 bp as described in detail by Wilkinson (1992). To assess the efficiency of probe labeling, dot blotting was performed as set out previously (van der Eerden et al. 2002b).

In situ hybridization and control hybridizations

We used the method described in detail previously (van der Eerden et al. 2002b) with some modifications. Briefly, sections were deparaffinized and incubated with 5 µg/ml (aromatase, type I and II 17β-HSD and STS) or 10 µg/ml (5α-reductase) proteinase K (Life Technologies, Breda, The Netherlands). After washing, hybridization was performed overnight at 50 °C (aromatase, type I 17β-HSD and STS) or 60 °C (type II 17β-HSD and 5α-reductase). The hybridization mixture consisted of 50% formamide, 2 × SSC, 1 × Denhardt solution, 10% dextran sulfate, 1 mg/ml yeast tRNA and the probe of interest (see Table 1). All probes contained a similar amount of incorporated DIG-UTP as assessed by dot blot. After hybridization, and RNase A (20 µg/ml) (Sigma) treatment, sections were blocked in sheep serum. Alkaline phosphatase-conjugated anti-DIG (1:1250) (Roche) was applied overnight at 4 °C and staining was performed in a Nitro Blue Tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt solution (Roche) varying from 6 h to overnight. Finally, the sections were counterstained with 0·8% Methyl Green (ERs) or 0·5% Neutral Red (enzymes), dehydrated and mounted in Euparal (Klinipath, Königsgen, Germany). Statements on the mRNA expression patterns over time and between the sexes were based on the analysis of multiple tissue sections of four animals (at least two sections of each animal) in each experimental group. Representative pictures of the growth plate sections were taken with a Nikon DXM 1200 digital camera using the same settings.

Immunohistochemistry

The immunohistochemical protocol performed was identical to that published earlier (van der Eerden et al. 2002b). The antibodies for ERα and ERβ (MC-20 and Y-19 respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) have both been extensively tested for their specificity in positive control tissues and by preincubation with corresponding peptides as negative controls (van der Eerden et al. 2002b). In short, after digestion with pepsin and blocking, sections were incubated overnight at 4 °C with the rabbit polyclonal MC-20 (1:50) or goat polyclonal Y-19 (1:50) as primary antibody in 0·5% blocking buffer. After a number of secondary antibody steps, staining was visualized with 3-amino-g-ethylcarbazole (0·2 mg/ml in acetate buffer pH 5·2 with 0·04% H2O2; Sigma) for 3 min. After counterstaining with hematoxylin, the sections were embedded in Aquamount (BDH, Poole, Dorset, UK). Statements on protein expression patterns over time and

### Table 1

<table>
<thead>
<tr>
<th>Probe</th>
<th>Vector</th>
<th>Size (bp)</th>
<th>Nucleotides</th>
<th>Concentration (pg/µl)</th>
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<td>pBluescript SK-</td>
<td>602</td>
<td>1–602</td>
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<tr>
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<td>1289</td>
<td>1–1289</td>
<td>S: 200, AS: 200</td>
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<tr>
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<td>344</td>
<td>991–1334</td>
<td>S: 133, AS: 200</td>
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<tr>
<td>STS</td>
<td>pBluescript SK-</td>
<td>252</td>
<td>472–723</td>
<td>S: 200, AS: 200</td>
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<tr>
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<td>1250–1737</td>
<td>S: 750, AS: 1500</td>
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<tr>
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<td>391</td>
<td>1–391</td>
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</tbody>
</table>
between the sexes were based on the analysis of multiple tissue sections of four animals (at least two sections per animal) in each experimental group. Pictures of growth plate sections were taken with a Nikon DXM 1200 digital camera using the same settings.

**Staining according to the Masson–Goldner method**

Two 5 μm thick mid-sagittal sections of the tibial metaphysis from all experimental groups (n=6) underwent trichrome staining according to the Masson–Goldner method (Clark 1981). Sections were deparaffinized and washed in distilled water for 5 min. Nuclei were stained in 1% Mayer’s hematoxylin (Merck, Darmstadt, Germany) for 40 s and directly washed for 10 min in running tap water. Next, sections were stained with Azophloxin (Merck) for 5 min and rinsed in 1% acetic acid. Then, 2% Orange G (Merck) solution was applied to the slides for 3 min followed by another rinse in 1% acetic acid. Counterstaining was performed with 0.2% Light Green (Sigma) for 15 min, followed by a rinse in 1% acetic acid. After dehydration, the sections were mounted in Histomount (National Diagnostics, Atlanta, GA, USA). Representative pictures of the tibial metaphyses just below the proximal growth plate were taken from all experimental groups. These were converted to grayscale images and black and white images, and from these TBV was determined and expressed as a percentage of total bone volume. Values were averaged ± S.D. and differences between groups were analyzed for significance, using the Student’s t-test. A P value less than 0.05 was considered significant.

**Results**

**Expression of ERα and ERβ mRNA and protein in the tibial metaphysis**

In order to identify bone cells in the rat tibial metaphysis, which express ERs, *in situ* hybridization with specific cRNA probes was performed on sections of the proximal tibiae from 1-, 4-, 7- and 16-week-old modeling female and male rats (n=4). Both ERα and ERβ mRNA were detected in osteoblasts, lining cells and osteoclasts during development as exemplified in a female rat at 4 weeks of age (ERα: Fig. 1A and B; ERβ: Fig. 1D and E). In osteocytes, we detected neither ERα nor ERβ mRNA. Control experiments using the respective sense probes did not reveal staining in any sections (Fig. 1C and F respectively). The expression of both ER mRNAs in bone did not change with time and did not differ between female and male rats. Immunohistochemically, the MC-20 (ERα) and Y-19 antibody (ERβ) were used to detect the ERs in the tibial metaphysis. The staining patterns for both ER proteins overlapped with the mRNA expression patterns. Some nuclear but predominantly cytoplasmic staining was observed in osteoblasts, lining cells and osteoclasts using both antibodies as exemplified in a 4-week-old female rat (ERα, Fig. 1G and H; ERβ, Fig. 1J and K). In addition, a few ERα- and ERβ-positive osteocytes were found (Fig. 1G and J and K respectively). Analysis of multiple tissue sections from each age group (n=4) and from both sexes, did not reveal a difference in immunostaining between the sexes and over time. Control experiments in which the first antibody was omitted or the sections were preincubated with blocking peptides resulted in absence of staining (Fig. 1I and L). In a previous study, we have extensively described the specificity of the probes and antibodies, using positive control tissues such as uterus and ovary (van der Eerden et al. 2002b).

**Expression of enzymes involved in sex steroid metabolism in the tibial metaphysis**

We next examined the mRNA expression of various enzymes involved in sex steroid metabolism at the same timepoints, using *in situ* hybridization (Fig. 2). No differences of the enzyme mRNA expression patterns were observed between females and males, based on the analysis of at least two tissue slides from each animal (n=4) in all age groups. The mRNAs for aromatase (Fig. 2A–D), type I (Fig. 2E–G) and type II 17β-HSD (Fig. 2H–K), STS (Fig. 2L–O) and type I 5α-reductase (Fig. 2P–S) were all expressed in the metaphysis during and after sexual maturation as exemplified in female rats at 7 (Fig. 2A, B, E, H, I, L, M, P and Q) and 16 weeks of age (Fig. 2C, D, F, J, K, N, O, R and S) respectively. In contrast, before sexual maturation at 1 and 4 weeks of age, none of the enzyme mRNAs were detected except STS, which was already present in 1-week-old rats onwards (data not shown). The mRNA expression of all enzymes was predominantly observed in osteoblasts (Fig. 2A, C, E, H, J, L, N, P and R) and osteoclasts (Fig. 2B, D, G, I, K, M, O, Q and S) and occasionally in osteocytes (Fig. 2A, L and Q). Type I 17β-HSD mRNA was only detected in osteoblasts and not in osteoclasts at 7 weeks of age (data not shown), whereas at 16 weeks of age osteoclasts were weakly positive (Fig. 2G). In control experiments, no staining was observed using sense cRNA probes for aromatase, type I and II 17β-HSD, STS and 5α-reductase (Fig. 2T–X respectively). We have recently described control experiments, demonstrating the specificity of the enzyme cRNA probes in uterus, ovary and prostate tissue (van der Eerden et al. 2002b).

**Effect of gonadectomy and E₂ supplementation on TBV before sexual maturation**

We showed that both ERs were expressed in the metaphysis before sexual maturation, while the various enzymes involved in sex steroid metabolism were not present. Taken together with observations that endogenous sex steroid levels are very low or undetected before
sexual maturation in the rat (Saksena & Lau 1979), we assessed the functional significance of the ERs in the metaphysis at this stage. Therefore, 26-day-old female and male rats were either gonadectomized or supplemented with E2. In general, rats enter the sexual maturation stage around day 30 (Tanner 1962), indicating that in our experiments rats were still sexually immature at the start of the experiment.

Gonadectomy of 26-day-old rats resulted in a lower TBV as assessed by Masson–Goldner staining in males \((8.85 \pm 1.91 \text{ vs } 18.1 \pm 7.72\% ; P=0.030)\) and a trend towards a lower TBV in females (Fig. 3A; \(7.62 \pm 3.18 \text{ vs } 13.3 \pm 4.80\% ; P=0.057)\) compared with controls. When E2 was administered to 26-day-old rats via slow-release pellets during 3 weeks, the opposite occurred; compared with placebo, a strong increase in TBV was evident in females (Fig. 3A; \(28.8 \pm 12.6 \text{ vs } 12.2 \pm 2.59\% ; P=0.042)\) and males (\(37.7 \pm 7.57 \text{ vs } 21.9 \pm 1.79\% ; P<0.001)\). Representative black and white images from each group of both experiments exemplify the histogram (Fig. 3B). OVX (Fig 3B, b) and orchidectomy (d) demonstrated lower TBV compared with sham-operated

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Figure 1 In situ hybridization and immunohistochemistry of ERα and β in the tibial metaphysis of the rat. ERα and β mRNA were expressed in lining cells, osteoblasts (A and D respectively) and in osteoclasts (B and E respectively) as exemplified in a 4-week-old rat. Both ERα and β mRNA were not detected in osteocytes (A and D respectively). Arrows indicate the different cell types. Sense hybridizations for ERα (C) and ERβ (F) demonstrated absence of signal in bone. Staining for ERα with the MC-20 antibody and for ERβ with the Y-19 antibody was observed in lining cells, osteoblasts (G and J respectively) and in osteoclasts (H and K respectively) as exemplified in a 4-week-old rat. Both ERα and β were occasionally detected in osteocytes (G and J and K respectively). Arrowheads indicate the different cell types. Preincubation of MC-20 or Y-19 antibody with a blocking peptide (I and L respectively), resulted in absence of staining. Bar represents 50 μm (A, C, D, F, G, I-L) or 10 μm (B, E, H). Abbreviations: OB, osteoblast; LC, lining cell; OCY, osteocyte; OC, osteoclast; BM, bone marrow.
animals (a and c respectively), whereas E₂ supplementation increased TBV in females (f) and males (h) compared with placebo (e and g respectively).

The TBV of sham-operated and placebo-control rats in the two experiments were not significantly different (females: 13.3 ± 4.80 vs 12.2 ± 2.59%, P = 0.69; males: 18.1 ± 7.72 vs 21.9 ± 1.79%, P = 0.43). As expected, TBV values of males in the control group were higher compared with females in both experiments but only significant in the E₂-supplementation experiment (gonadectomy experiment: 18.1 ± 7.72 vs 13.3 ± 4.80%, P = 0.21; E₂-supplementation experiment: 21.9 ± 1.79% vs 12.2 ± 2.59%, P < 0.01).

The effects of gonadectomy and E₂ supplementation on other parameters in these animals, such as longitudinal growth, body weight gain, growth plate thickness and development of the gonads are described elsewhere (van der Eerden et al. 2002a).

**Discussion**

Several studies have established that sex steroids can exert direct effects on bone cells, since receptors for estrogen and androgen have been demonstrated in lining cells, osteoblasts, osteocytes and osteoclasts. To date, however, no studies have thoroughly investigated the expression of both ERs before, during and after sexual maturation in bone. Furthermore, it is unclear whether metabolism of sex steroids in bone cells can occur at these timepoints. To address these issues, we have performed a systematic survey of ERα and β mRNA and protein expression in the tibial metaphysis, using in situ hybridization and immunohistochemistry on tibial sections from 1-, 4-, 7- and 16-week-old female and male rats. Furthermore, we studied the mRNA expression of various enzymes in sex steroid metabolism, aromatase, type I and II 17β-HSD, STS and 5α-reductase in the same age groups. These stages are characterized by fast growth and bone mass accrual before (1 and 4 weeks) and during sexual maturation (7 weeks), followed by bone modeling at a lower pace after sexual maturation (16 weeks). Not until 9 months of age does bone modeling come to an end and bone remodeling becomes predominant in rats (Wang et al. 2001).

We demonstrate that both ERs are present in osteoblasts, osteoclasts and some osteocytes of the tibial metaphysis irrespectively of age and gender. The mRNA of various key enzymes involved in sex steroid metabolism were also present in bone-forming and -degrading cells, however, only from week 7 and onwards, with the exception of STS mRNA, which was present at all timepoints.

ERα and β mRNA and protein were predominantly expressed in lining cells, osteoblasts and osteoclasts and occasionally in osteocytes, irrespectively of age and gender. Expression of ERα in cultured human osteoblast-like cells and osteoclasts is well documented (Arts et al. 1997, Onoe et al. 1997, Gruber et al. 1999), but localization of ERα in bone tissue sections is less well described. Kusec et al. (1998) found ERα mRNA in osteoblasts of rabbit and human bone only, whereas ERα has been shown in osteocytes of human, pig and guinea pig (Braidman et al. 1995). Another study reported very low levels of ERα mRNA in human osteocytes (Hoyland et al. 1997). Apparently, localization of ERα on osteoblasts and osteocytes, but in our hands also osteoclasts, depends on the species, protocol and/or the antibody used to detect the receptor.

ERβ mRNA expression was reported in osteoblasts of neonatal rat bone (Windahl et al. 2000), whereas the protein was present in mouse and human osteoblasts, osteoclasts and osteocytes (Vidal et al. 1999, Braidman et al. 2001), which agrees well with our findings. Our expression data imply that estrogen can exert its regulatory role in bone modeling directly on both bone-forming and -degrading cells in the female and male rat during development starting from 1 week after birth. Expression of both ERs did not seem to alter during sexual maturation, suggesting that regulation of the ERs by rising levels of circulating estrogen might not occur in metaphyseal bone at least at the mRNA and protein level. However, other more quantitative techniques should confirm this statement.

Aromatase, type I and II 17β-HSD, STS and 5α-reductase mRNA were also present in the metaphyseal bone of the rat. The enzymes were mainly detected in osteoblasts and osteoclasts but occasionally also in osteocytes, irrespective of gender. Interestingly, all enzyme mRNAs were expressed from sexual maturation (7 weeks onwards).

**Figure 2** In situ hybridization of aromatase, type I and II 17β-HSD, STS and 5α-reductase mRNA in the tibial metaphyses of the rat. In situ hybridization demonstrating aromatase (A–D), type I and II 17β-HSD (E–G and H–K respectively), STS (L–O) and 5α-reductase (P–S) mRNA in sections of tibial growth plates from female and male rats. Aromatase, type I 17β-HSD, type II 17β-HSD, STS and 5α-reductase mRNA were all expressed in females at 7 (A and B; E and H; I and L; and M and P and Q) and 16 weeks of age (C and D; F; J and K; N and O; S respectively) and was predominantly found in osteoblasts (A and C; E; H; I; L and M; and P and Q and R; respectively), osteoclasts (B and D; G and I; K and M; and O; Q and S respectively) and occasionally in osteocytes (indicated with arrows in A, L and Q). Control hybridizations with sense RNA probes for aromatase (T) type I and II 17β-HSD (U and V respectively), STS (W) and 5α-reductase (X) showed no signal. Bar represents 50 μm (L–X) or 10 μm (A–K, L–S). Abbreviations: OB, osteoblast; LC, lining cell; OCY, osteocyte; OC, osteoclast; BM, bone marrow.
of age) and thereafter (16 weeks of age). In contrast, none of these enzymes was detected before sexual maturation (1 and 4 weeks of age), except for STS, which was already present from 1 week onwards.

Combining the findings in this study and of a recent paper by us (van der Eerden et al. 2002b), we have demonstrated co-expression of both ERs, AR, and various enzymes involved in sex steroid metabolism, suggesting that intracrinology may take place in metaphysial bone predominantly from the onset of sexual maturation onwards in the rat. Even before sexual maturation, low levels of circulating E1-S may be converted by the presence of STS. Local sex steroid metabolism may therefore play a role in the beneficial effects of E2 on bone mass accrual, possibly by intracrinology.

Our findings are in line with other reports, indicating that intracrinology occurs in various target tissues of sex steroids including several brain regions, such as the amygdala and the cortex, and cultured hypothalamic neurons in the rat (Jacobson et al. 1997, Negri-Cesi et al. 2001). These and our findings are in disagreement with a recent paper claiming that sex steroids in rodents are exclusively formed in the gonads (Labrie et al. 2000).

Various other studies have reported expression and bioactivity of enzymes in human and rat bone cells, although these studies have mainly focused on the aging skeleton and/or osteoblastic cell lines and not on the modeling skeleton around sexual maturation (Dong et al. 1998, Eyre et al. 1998, Saito & Yanaihara 1998, Feix et al. 2001). Sasano et al. (1997) have demonstrated aromatase and type I 17β-HSD protein as well as aromatase mRNA in osteoblasts and lining cells in bone derived from middle-aged or older adults. Only very recently, aromatase protein was found in osteoblasts and osteoclasts from human femoral material that was obtained from an adolescent male and female undergoing epiphysectomy to counter premature growth plate fusion in the opposite leg (Oz et al. 2001). To date, no other reports have localized aromatase in metaphysial bone cells, nor any other enzyme investigated in this study.

By upregulating the expression/activity of various enzymes involved in estrogens metabolism around sexual maturation/puberty, high amounts of active estrogens could be formed locally. This may result in the increased rate of bone maturation that is evident during sexual maturation in rodents and during puberty in man. In support of this, in ERα- and aromatase-deficient patients, pubertal bone maturation and growth plate closure fail to occur (Smith et al. 1994, Morishima et al. 1995).

We should note, however, that we only examined mRNA expression of a number of enzymes involved in estrogen metabolism. Future studies should reveal whether this expression pattern associates with protein expression and enzyme activity. In a recent study, we observed an excellent correlation between mRNA expression and enzymatic activity in rat tibial chondrocytes of aromatase, type I 17β-HSD, and STS (van der Eerden et al. 2002c).

As both ERs were expressed in metaphysial bone cells during sexual maturation, independently of the rising circulating levels of sex steroids, whereas the enzymes involved in sexual maturation were present from sexual maturation onwards, we investigated the functional relevance of the ERs in the metaphysis at this stage. To address this, the effect of gonadectomy or E2 supplementation on TBV was studied in 26-day-old female and male rats, which were sexually immature at the start of the experiment, whereas at the end of the experiment (3 weeks later) control rats were sexually maturing. Therefore, we cannot completely rule out sexual maturation effects on measurements in the metaphysis of control rats at the end of the experiment. However, as gonadectomized rats do not sexually mature due to the absence of sex steroids, whereas rats receiving E2 contain very high estrogen levels (approximately 1000-fold above endogenous levels), the interference of endogenous estrogen levels will be minimal.

Compared with controls, TBV was reduced (trend towards reduction in females) following gonadectomy and increased following E2 supplementation in both sexes. The observed findings coincide with other studies in rats before and during early sexual maturation, in which bone loss was observed in mandibular condyles and in the temporomandibular joint (Okuda et al. 1996, Yamashiro & Takano-Yamamoto 1998). Most studies, however, were performed in aged animals as models for postmenopausal bone loss, when bone remodeling instead of bone modeling is predominant (Wronska et al. 1986, Bagi et al. 1993, Turner et al. 1994b). The lack of a significant effect in females might be due to the fact that only during the latter half of the experiment are E2 levels different between sham and OVX animals. As a consequence, the time period during which E2 levels were low or absent was too short to observe a more pronounced effect on bone loss. Since E2 levels are low in males, the observed significant bone

![Figure 3](https://www.endocrinology.org)
loss may reflect reduced androgen signaling, rather than reduced estrogen signaling.

In our study, supraphysiological doses (23 µg/day) of E$_2$ in 26-day-old intact rats dramatically increased TBV, in both females and males. This agrees well with other studies in rats during (Wakley et al. 1997) and just after sexual maturation (Tobias et al. 1991), although much higher doses were used compared with that in our study (~200 vs 23 µg/day). The anabolic effect of E$_2$ has been associated either with inhibited bone resorption, as observed in 7-week-old OVX rats (Turner et al. 1994a,b) or 2-month-old male rats (Wakley et al. 1997), or with increased bone formation, reported in 3-month-old female rats (Tobias et al. 1991). Our data do not provide an answer to whether the observed increase in TBV is due to enhanced formation or a reduction of resorption. The data also indicate that estrogen is not only important for bone mass accrual in female rats, but also in males. Clinical findings in male patients with an inactivating mutation of the ER gene and aromatase deficiency (Smith et al. 1997) have shown that this also applies to humans.

Our data suggest that changes in the level of estrogen may have major effects on bone mass accrual, even just before sexual maturation when endogenous levels of estrogen are low or undetected (Sakena & Lau 1979). At these timepoints, E$_2$ may have direct effects on bone cells themselves, since they express ERs, even before sexual maturation. However, E$_2$ may also exert indirect effects on bone, e.g. by influencing the somatotropic axis. Indeed, it has been shown that E$_2$ regulates the pulse and amplitude of growth hormone (GH) secretion in humans (Veldhuis 1998). Furthermore, both GH and insulin-like growth factor-I have anabolic effects on bone (Ohslnson et al. 2000). Pro-apoptotic effects of E$_2$ have been reported for osteoclasts, and anti-apoptotic effects for osteoblasts and osteocytes, which play a role in prevention of postmenopausal bone loss by E$_2$ (Greenfield et al. 1999, Koutsteni et al. 2001). Whether these mechanisms contribute to the modeling skeleton is less clear and will be the subject of future research.

In conclusion, whereas both ERs are present in rat metaphyseal bone from birth onwards, various key enzymes involved in sex steroid metabolism are first present from sexual maturation onwards. This suggests that in metaphyseal bone the local formation of the most potent sex-steroids (E$_2$ and testosterone) as well as intracrinology may play a significant role in bone mass accrual and maintenance starting from sexual maturation and thereafter.

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ERs and enzymes during sexual maturation


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