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Testosterone Prevents Orchidectomy-Induced Bone Loss in Estrogen Receptor-α Knockout Mice

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To examine the role of the estrogen receptor-α (ERα) during male skeletal development, bone density and structure of aged ERαKO mice and wild-type (WT) littermates were analyzed and skeletal changes in response to sex steroid deficiency and replacement were also studied. In comparison to WT, ERαKO mice had smaller and thinner bones, arguing for a direct role of ERα to obtain full skeletal size in male mice. However, male ERαKO mice had significantly more trabecular bone as assessed both by pQCT and histomorphometry, indicating that ERα is not essential to maintain cancellous bone mass. Six weeks following orchidectomy (ORX), both WT and ERαKO mice showed high-turnover osteoporosis as revealed by increases in serum osteocalcin and decreases in trabecular (−38% and −58% in WT and ERαKO, respectively) and cortical bone density (−5% and −4% in WT and ERαKO, respectively). Administration of testosterone propionate (T, 5 mg/kg/day) completely prevented bone loss both in ERαKO and in WT mice. As expected, estradiol (E2, 60 μg/kg/day) replacement did not prevent cancellous bone loss in ORX ERαKO mice. However, E2 stimulated bone formation at the endocortical surface in ORX ERαKO, suggesting that osteoblasts may respond to nonERα-mediated estrogen action. In conclusion, although functional ERα may play a significant role during male skeletal development, this receptor does not seem essential for androgen-mediated skeletal maintenance in older male mice.

Key Words: **androgens; estrogens; estrogen receptor-α; mice; orchidectomy; bone.**

Androgens are crucial for both skeletal development and maintenance in men and male rodents. The effects of androgens may be mediated directly by the androgen receptor (AR) or indirectly via aromatization of androgens into estrogens and subsequent stimulation of estrogen receptor-α (ERα) and/or estrogen receptor-β (ERβ). That estrogens may indeed play an important role in male skeletal growth was suggested by studies using an aromatase inhibitor, showing delayed skeletal modeling in growing male rats (1). Moreover, both aromatase knockout (ArKO) (2) and estrogen receptor-α knockout (ERαKO) mice (3) show impaired skeletal development. Also in humans, men with mutations in either the ERα (4) or the aromatase gene (5, 6) show delayed skeletal maturation. Aromatization of androgens may also be important for skeletal maintenance after growth. Indeed, administration of an aromatase inhibitor induces bone loss in aged male rats (7). Additionally, estrogen (8, 9) and selective estrogen receptor modulators (10, 11) prevent orchidectomy-induced bone loss in rodents. In elderly men, bone density seems to correlate more closely with estrogen than with androgen concentrations (12–14). In these men, estrogen also appears to be the dominant sex steroid regulating bone resorption (15). Finally, impaired ERα expression in osteoblasts and osteocytes in men suffering from idiopathic osteoporosis has been reported (16).

ER-mediated androgen action may, however, not explain all aspects of androgen effects on the male skeleton. This is illustrated by observations that administration of nonaromatizable androgens prevents castration-induced bone loss in adult rats (8). Furthermore, androgen-resistant rats (17) have decreased longitudinal and radial bone growth.

The ERαKO model is a unique model to study the relevance of ERα- versus nonERα-mediated androgen

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action on the male skeleton. Aged (7.5-month-old) ERαKO mice versus wild-type (WT) mice can be used to analyze the role of ERα during male skeletal development and maintenance. Furthermore, comparing the skeletal changes in these ERαKO mice in response to sex steroid deficiency and replacement (both androgens and estrogens) allows the evaluation of nonERα-mediated androgen and estrogen action.

MATERIALS AND METHODS

Animals. Aged, six-month-old male ERαKO mice and their wild-type (WT) littermates, weighing approximately 30 g, were obtained from an in-house breeding strain (C57BL/6J background). The ERαKO animals were originally purchased from Dr. K. Korach (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC) (18). The animals were housed individually in an air-conditioned room (21 ± 2°C) with a daily light schedule of 14 h light and 10 h near dark. During the experiment, the mice had free access to tap water and were fed a diet containing 1.14% calcium, 0.8% phosphorus, 2200 IU/kg vitamin D3, 6.2% fat and 25% protein (Hope Farms, Linschoten, The Netherlands). All animal procedures were approved by the local Animal Ethics Committee.

Experimental design. Thirty six-month-old male ERαKO mice were divided into five groups ($n = 6$) and twenty age-matched male WT mice were divided into four groups ($n = 5$), according to a randomized block design using body weight as a selection parameter. One group of ERαKO mice (Base) was sacrificed at the start of the experiment, three groups were orchidectomized and the remaining group was sham-operated using isoflurane anaesthesia. One group of WT mice was sham-operated and the three other groups were orchidectomized using the same anaesthesia. Both the sham-operated mice (Sham) and the control orchidectomized group (ORX) were treated daily with 0.1 ml vehicle, whereas the other orchidectomized mice received either 60 μg/kg 17β-estradiol (ORX+E2) or 5 mg/kg testosterone propionate (ORX+T), once daily subcutaneously (sc), during an experimental period of 6 weeks. All animals were weighed weekly and were given an intraperitoneal (ip) injection of the fluorochrome calcein (16 mg/kg, Sigma Chemical Co., St. Louis, MO) 9 and 2 days before sacrifice. At the end of the study, the animals were anaesthetized with ether and blood was taken from the retroorbital venous plexus. Serum specimens were collected and stored at −20°C until assay. The efficiency of ORX and sex steroid replacement was confirmed by measurement of seminal vesicles and ventral prostate weights as well as serum T levels (data not shown). The tibiae and femora were dissected and cleaned from surrounding tissue. The left tibia and femur were immersed in Burkhardt's fixative for 24 h at 4°C and subsequently kept in 100% ethanol until histomorphometric analysis. The right femur was used for measurement of cortical and trabecular volumetric density and geometry by pQCT *ex vivo.*

Assays. Serum testosterone was measured on an Immulite 1000 auto-analyzer (Diagnostic Products Corp., Los Angeles, CA). Serum alkaline phosphatase activity was determined by standard methods using an auto-analyzer. Serum osteocalcin, a marker of bone formation, was quantitated by a radioimmunoassay as previously described (19). Intra- and interassay variations were 5.9 and 5.2% respectively.

Static and dynamic histomorphometry. The undecalcified left tibiae were embedded in methylmetacrylate, and 4 μm thick longitudinal sections were cut with a rotation microtome (RM 2155 Autocut, Leica, Heidelberg, Germany) using a tungsten carbide blade (Leica, Nussloch, Germany). The sections were stained by a modified Goldner technique and subjected to static histomorphometric analysis. All measurements were performed in the proximal tibial metaphysis with a Kontron Image Analyzing Computer (KS 400 3.00, Kontron Bildanalyze, Munich, Germany). The trabecular bone volume (B.Ar/T.Ar, as a percentage of total area) was assessed in the secondary spongiosa of at least three sections, each 60 μm apart. In each section, three consecutive fields (tissue area of 1 mm^2) were measured along the vertical axis of the central metaphysis, starting 0.25 mm from the distal end of the growth plate, thereby excluding the endocortical surface. The osteoid surface (O.Pm/B.Pm, as a percentage of total trabecular bone perimeter) was quantitated on three sections, each 60 μm apart, in the secondary spongiosa of the metaphysis, starting 0.25 mm from the distal end of the growth plate and excluding the endocortical surface.

After fixation, the left femora were embedded undecalcified in methylmethacrylate, as described previously (20). In the middiaphysis of the left femur, 200-μm-thick sections were prepared with a precision band saw (Exakt, Norderstedt, Germany) perpendicularly to the long axis of the bone. Subsequently, the sections were ground to a final thickness of 20 μm using a micro-grinding system (Exakt), and left unstained or stained with toluidine blue. Structural parameters were measured with an automatic image analysis system (VIDAS, C. Zeiss, Oberkochen, Germany) connected to a Zeiss stereo microscope via a TV-camera. The image analysis system automatically determined the total cross-sectional area (Tt.Ar), the cortical bone area (Ct.Ar), the marrow area (Ma.Ar), and the endocortical (Ec.Pm) and periosteal bone perimeter (Ps.Pm). Measurements of fluorochrome-based histomorphometric parameters were made using a semiautomatic system (Videoplan, C. Zeiss) and a Zeiss Axioskop microscope with a drawing attachment. Dynamic fluorochrome-based parameters were measured on unstained sections. Double-labeled bone perimeter was measured at ×100. The mineral apposition rate (MAR) was measured at ×400, sampling each double label every 50 μm. The mineralizing perimeter (M.Pm/B.Pm) was defined as the percentage of fluorochrome double-labeled bone perimeter. The periosteal and endocortical bone formation rate (BFR/B.Pm) was calculated by multiplying the mineralizing perimeter with the respective mineral apposition rate. All histomorphometric parameters were reported according to the recommended American Society for Bone and Mineral Research nomenclature (21).

Peripheral quantitative computed tomography. Trabecular and cortical bone mineral content (BMC) and density (BMD) and the geometry of the femur were assessed by pQCT (XCT Research M^+, Norland Medical Systems, Inc., Fort Atkinson, WI), using software version 5.40. Slices of 0.3 mm thickness were scanned using a voxel size of 0.07 mm. One scan was taken 2 mm from the distal end of the femur, using contmode 1, peelmode 20 and an attenuation threshold of 0.280 cm^{-1}. The trabecular bone region was defined by setting an inner area to 25% of the total cross-sectional area. These metaphyseal pQCT scans of the femur were performed to measure trabecular volumetric density. Another scan was taken 7 mm from the distal end of the femur (an area containing mostly cortical bone) using contmode 1, separation mode 1 and an attenuation setting of 0.710 cm^{-1}. These middiaphyseal pQCT scans were performed to determine cortical volumetric density, cortical thickness, periosteal perimeter and endocortical perimeter. The precision and stability of the measurements were evaluated periodically, and the coefficient of variation was <1.2%.

Statistical analysis. Data analysis was performed using a statistical software program (NCSS, Kaysville, UT). All data are expressed as mean ± standard error of the mean (SEM). Significance of difference between wild type and ERαKO mice was assessed by a two-sample *t* test. One-way analysis of variance (ANOVA) was carried out to detect overall differences and if *P* was less than 0.05, was followed by Fisher's least-significance-difference multiple-comparison test to calculate intergroup differences.

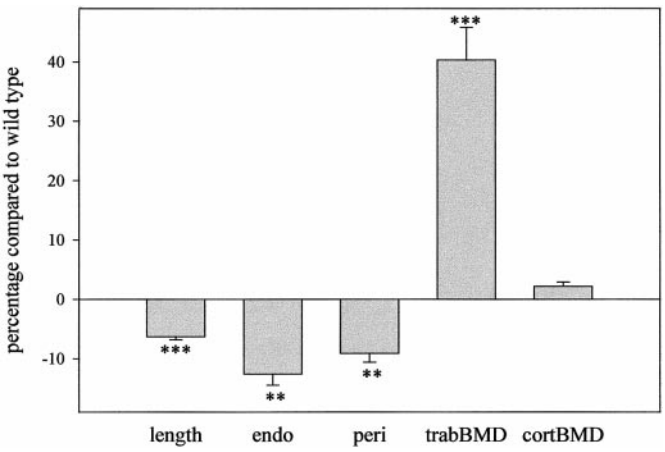

FIG. 1. Femoral length (length), and endocortical (endo) and periosteal (peri) perimeters, trabecular (trabBMD) and cortical (cortBMD) bone mineral density, as measured by pQCT, in 7.5-month-old male Sham-operated (Sham) ERαKO mice, expressed as a percentage of the respective Sham WT parameters. Data are expressed as mean ± SEM, $n = 4–6$ mice per group. $^{**}P < 0.01$ vs Sham WT, $^{***}P < 0.001$ vs Sham WT.

RESULTS

Comparison of Male ERαKO Mice with Age-Matched WT Mice

Sham ERαKO mice had both smaller and thinner femora compared to Sham WT mice (Fig. 1), despite similar body weights (Sham ERαKO 34.7 ± 1.1 g vs Sham WT 35.5 ± 0.8 g). Significantly smaller periosteal (−9%) and endocortical (−13%) perimeters resulted in reduced cortical and marrow areas (Fig. 1, Table 1), but similar cortical thickness. Furthermore, endocortical and periosteal MAR and BFR were not significantly different (Table 1). Serum osteocalcin tended to be lower in Sham ERαKO mice (Fig. 3A), whereas serum alkaline phosphatase levels were not different (Fig. 3B). Cortical BMD was not affected, but trabecular BMD of the distal femur was 40% higher in Sham ERαKO mice (Fig. 1). Accordingly, trabecular bone volume of the proximal tibia was also significantly higher (+175%) (Table 1). The higher testosterone concentrations measured in ERαKO mice (Sham ERαKO 11.4 ± 2.6 ng/ml vs Sham WT 7.7 ± 2.1 ng/ml) may be relevant in this respect.

Effects of Orchidectomy versus Sex Steroid Replacement

Trabecular BMD of the distal femur was significantly reduced following castration in both ERαKO (−58%, also compared to Base, data not shown) and WT mice (−38%) (Fig. 2A). This bone loss was confirmed by a comparable decrease of trabecular bone volume (−79%) in the proximal tibia (measured only in ERαKO mice) (Table 1). Cortical BMD followed a similar pattern in both ORX groups as reflected by significant decreases both in ERαKO (−4%) and WT mice

TABLE 1

Histomorphometric Analysis

	WT Sham	ERαKO Sham	WT ORX	ERαKO ORX	WT ORX+T	ERαKO ORX+T	WT ORX+E2	ERαKO ORX+E2
Proximal tibial metaphysis								
B.Ar/T.Ar (%)	2.72 ± 0.40	7.47 ± 1.01*	NM	1.55 ± 0.62†	NM	6.59 ± 0.51‡	33.81 ± 5.01	2.81 ± 0.95
O.Pm/B.Pm (%)	1.46 ± 0.75	0.53 ± 0.15	NM	10.29 ± 0.88†	NM	0.66 ± 0.16‡	3.76 ± 1.31	8.32 ± 0.91‡
Femoral middiaphysis								
Ps.Pm (mm)	5.54 ± 0.12	5.02 ± 0.10*	6.02 ± 0.44	5.10 ± 0.03	5.53 ± 0.17	5.13 ± 0.10	5.86 ± 0.47	5.18 ± 0.15
Ec.Pm (mm)	4.47 ± 0.17	3.67 ± 0.16*	4.37 ± 0.11	4.37 ± 0.51	4.37 ± 0.25	4.57 ± 0.71	3.64 ± 0.12‡	3.53 ± 0.30
Ct.Ar (mm^2)	0.93 ± 0.03	0.86 ± 0.02	0.91 ± 0.05	0.78 ± 0.03	0.92 ± 0.06	0.85 ± 0.04	1.16 ± 0.05‡	0.98 ± 0.05
Ma.Ar (mm^2)	1.14 ± 0.08	0.81 ± 0.05*	1.15 ± 0.03	0.91 ± 0.02	1.12 ± 0.06	0.93 ± 0.06	0.88 ± 0.08	0.75 ± 0.06
Tt.Ar (mm^2)	2.07 ± 0.09	1.67 ± 0.06*	2.07 ± 0.05	1.69 ± 0.03	2.04 ± 0.12	1.78 ± 0.08	2.04 ± 0.08	1.73 ± 0.10
Ec.M.Pm/B.Pm (%)	15.9 ± 5.8	16.1 ± 4.7	32.5 ± 10.9	43.1 ± 6.6†	5.5 ± 1.4‡	13.2 ± 9.8‡	77.1 ± 5.1‡	80.2 ± 2.6‡
Ec.MAR (μm/day)	0.89 ± 0.09	0.60 ± 0.05*	0.76 ± 0.02	0.88 ± 0.11	1.06 ± 0.16	1.22 ± 0.43	1.81 ± 0.12‡	1.27 ± 0.11
Ec.BFR/B.Pm ($\mu m^2/\mu m$/day)	0.15 ± 0.07	0.10 ± 0.03	0.25 ± 0.09	0.38 ± 0.06†	0.06 ± 0.02‡	0.14 ± 0.09‡	1.41 ± 0.17‡	1.01 ± 0.08‡
Ps.M.Pm/B.Pm (%)	24.2 ± 6.5	20.7 ± 10.0	22.7 ± 7.6	28.7 ± 7.7	7.4 ± 2.4	11.9 ± 5.1	42.6 ± 5.5‡	48.4 ± 4.5
Ps.MAR (μm/day)	0.53 ± 0.07	0.65 ± 0.08	0.61 ± 0.07	0.62 ± 0.05	0.78 ± 0.26	0.53 ± 0.07	0.77 ± 0.07	0.64 ± 0.02
Ps.BFR/B.Pm ($\mu m^2/\mu m$/day)	0.14 ± 0.06	0.17 ± 0.10	0.13 ± 0.04	0.19 ± 0.06	0.04 ± 0.01	0.07 ± 0.04	0.34 ± 0.06‡	0.31 ± 0.04

Note. Histomorphometric analysis of longitudinal sections of the proximal tibial metaphysis and cross-sections of the femoral middiaphysis in different experimental groups of both WT and ERαKO mice: sham-operated mice (Sham), orchidectomized mice (ORX), and orchidectomized mice treated with either testosterone propionate (ORX+T) or 17β-estradiol (ORX+ E2) during an experimental period of 6 weeks. Data are expressed as mean ± SEM, $n = 4–6$ mice per group. * $P < 0.05$ vs WT Sham; † $P < 0.05$ vs respective Sham; ‡ $P < 0.05$ vs respective ORX; NM, not measured. All other abbreviations are explained under Materials and Methods.

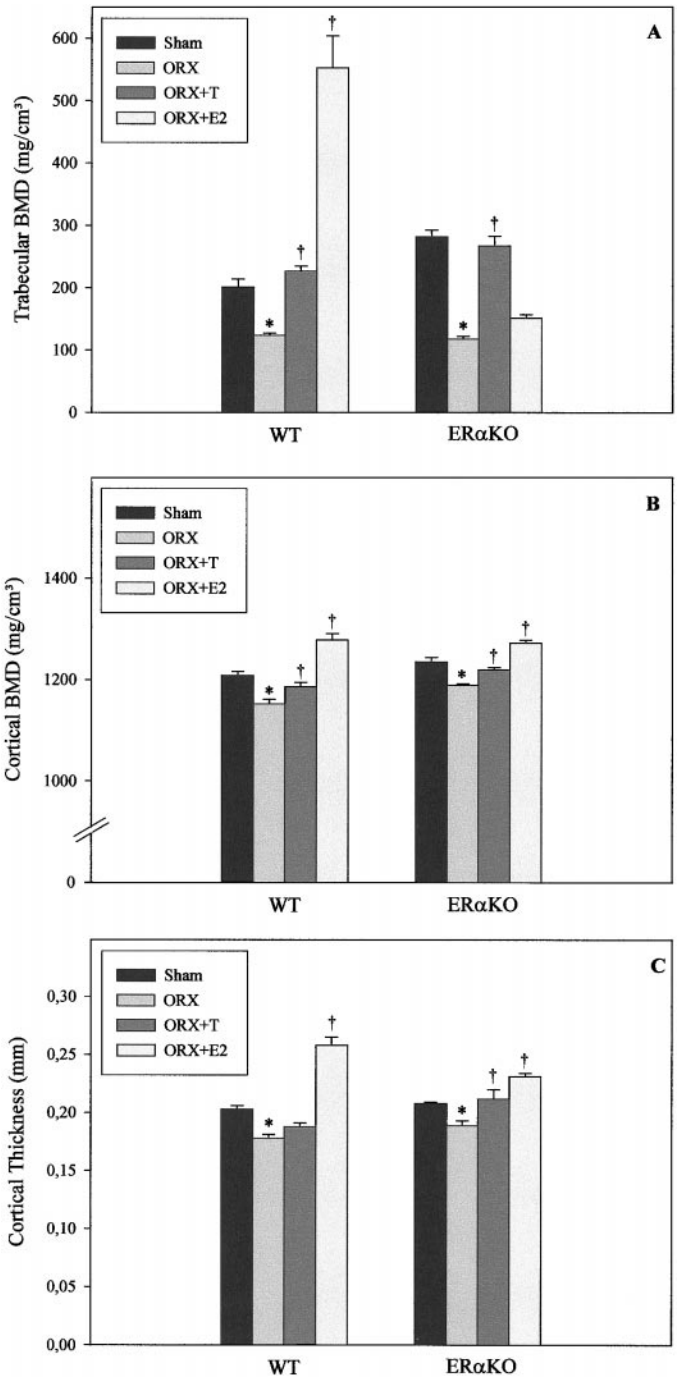

FIG. 2. Trabecular (A) and cortical (B) BMD, and cortical thickness (C) of the femur, as measured by pQCT, in different experimental groups of both male WT and ERαKO mice: sham-operated mice (Sham), orchidectomized mice (ORX), and orchidectomized mice treated with either testosterone propionate (ORX+T) or 17β-estradiol (ORX+E2) during an experimental period of 6 weeks. Data are expressed as mean ± SEM, $n = 4–6$ mice per group. *$P < 0.05$ vs respective Sham, †$P < 0.05$ vs respective ORX.

(−5%) (Fig. 2B). Cortical thickness was also reduced to the same extent in both groups following castration (Fig. 2C), due to a non-significant increase of endocortical perimeter without concomitant changes in periosteal perimeter (Table 1). These changes in bone density and structure were associated with an increased bone turnover rate, characterized by significantly increased serum osteocalcin levels both in ORX ERαKO (+237%) and WT mice (+84%) (Fig. 3A) and an increased osteoid surface (Table 1). Serum alkaline phosphatase levels, however, did not change following ORX in either ERαKO or WT mice (Fig. 3B). An increased endocortical BFR in both ERαKO and WT mice, resulting from an increase in endocortical mineralizing perimeter, completed the picture of a high bone turnover state in ORX mice (Table 1). The periosteal MAR and BFR were not different in both ORX groups compared to their respective Sham (Table 1).

Administration of T completely prevented loss of trabecular BMD (Fig. 2A), cortical BMD (Fig. 2B), and cortical thickness (Fig. 2C) in the femur and trabecular

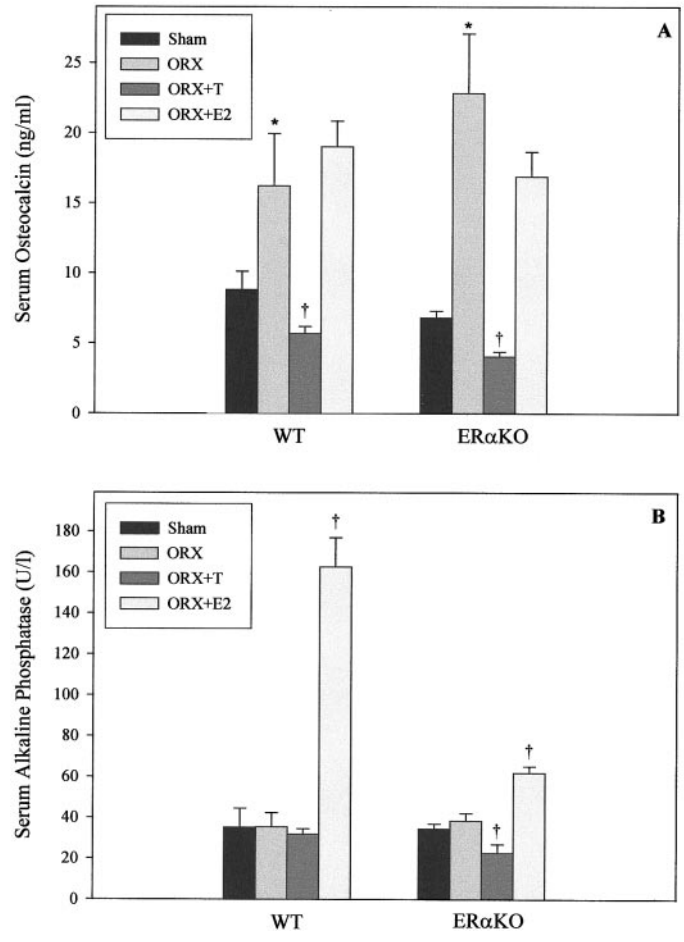

FIG. 3. Serum osteocalcin (A) and serum alkaline phosphatase (B) in different experimental groups of both male WT and ERαKO mice: sham-operated mice (Sham), orchidectomized mice (ORX), and orchidectomized mice treated with either testosterone propionate (ORX+T) or 17β-estradiol (ORX+E2) during an experimental period of 6 weeks. Data are expressed as mean ± SEM, $n = 4–6$ mice per group. *$P < 0.05$ vs respective Sham, †$P < 0.05$ vs respective ORX.

bone volume in the tibia (Table 1). This bone-sparing effect of T was reflected by decreased indices of bone turnover: serum osteocalcin was significantly lower both in ORX ERαKO and WT mice treated with T (Fig. 3A). Serum alkaline phosphatase was only significantly decreased in T-treated ORX ERαKO mice (Fig. 3B). Accordingly, the endocortical BFR was significantly lower following T replacement in ERαKO mice as well as in WT mice, due to a reduced endocortical mineralizing perimeter (Table 1). Furthermore, osteoid surface was decreased after T administration (measured only in ERαKO mice) (Table 1). T had no effect on the periosteal MAR and BFR in both ORX groups (Table 1).

Treatment of ORX WT mice with E2 increased trabecular BMD more than twofold (Fig. 2A). Histomorphometric analysis of the proximal tibia confirmed this osteosclerosis, showing a more than tenfold increase of trabecular bone volume (Table 1). Furthermore, the anabolic action of E2 not only increased cortical BMD (Fig. 2B) but also thickness (Fig. 2C) due to a significant reduction of the endocortical perimeter (Table 1). In addition, femoral length slightly increased in E2-treated ORX WT mice (WT ORX+E2 15.34 $\pm$ 0.14 mm vs WT ORX 15.00 $\pm$ 0.03 mm, $P = 0.05$). The overall effect of E2 was due to new bone formation as reflected by remarkably high levels of serum alkaline phosphatase levels (Fig. 3B), whereas serum osteocalcin remained in the range of ORX WT mice (Fig. 3A). Histomorphometric parameters confirmed E2-induced bone formation, showing significantly increased endocortical and periosteal BFR (Table 1).

As expected, ORX ERαKO mice were resistant to E2 action on cancellous bone: trabecular bone loss was not prevented by E2 in these mice (Fig. 2A, Table 1). Surprisingly, E2 protected against cortical bone loss (Fig. 2B) and thinning (Fig. 2C). This effect of E2 on cortical bone in ERαKO mice was accompanied both by high serum alkaline phosphatase levels (Fig. 3B) and a significantly increased bone formation at the endocortical surface (Table 1). The periosteal BFR was also increased, although significance was not reached (Table 1). Serum osteocalcin concentrations remained in the range of ORX ERαKO mice (Fig. 3A). Figure 4 gives an overview of the characteristic histological changes in the proximal tibial metaphysis in different experimental groups of male ERαKO mice.

DISCUSSION

ERαKO mice have a bone phenotype characterized by smaller and thinner bones compared to WT mice. This is in line with an earlier study (3), describing decreased longitudinal growth and impaired cortical modeling in young and adult ERαKO mice. In our study, bone turnover, as determined by serum osteocalcin and endocortical and periosteal BFR, was not lower in aged ERαKO mice as compared to WT mice. The observed delay of both longitudinal and radial skeletal growth must therefore have occurred earlier during development. This assumption of a delayed skeletal maturation is supported by earlier reported decreases of the skeletal growth factor insulin-like growth factor-I (IGF-I) (3). Similar low serum IGF-I concentrations, associated with reduced cortical modeling, were observed in male rats treated with an aromatase inhibitor (1). Taken together, the available data suggest an essential role for ERα in skeletal growth of male mice. Therefore, some of the stimulatory effects of androgens on skeletal growth may depend on ERα stimulation.

In contrast with the observed delay of skeletal maturation in ERαKO mice, trabecular bone density of the femur was not decreased but even up to 40% higher, indicating that ERα is not essential for the acquisition or maintenance of cancellous bone mass in male mice. Earlier studies, in younger mice, had already provided evidence that trabecular bone mass is not decreased in male ERαKO mice (3). Interestingly, male ERαKO mice have increased serum levels of testosterone (22). Based on these observations, it is tempting to speculate that continuous stimulation by elevated T levels may increase cancellous bone mass. This hypothesis assumes that T action on trabecular bone in these ERαKO mice is mediated via either the AR or ERβ. The latter possibility is unlikely, since male ERβKO mice have no bone phenotype (3, 23). It would seem, therefore, that the AR is essential for the acquisition and maintenance of cancellous bone mass in male mice.

In order to test the hypothesis that T may have a bone-protective action that is not mediated by ERα, we evaluated the skeletal changes in response to orchidectomy, both with or without T replacement, in ERαKO mice and WT. As expected, androgen deficiency in WT mice induced a characteristic rise in bone turnover, resulting in loss of trabecular and cortical bone density, and cortical thinning. These findings are in line with previous reports of androgen deficiency in male rats (8, 24) and mice (9, 25) and are similar to the well-documented osteopenia in hypogonadal men (26). Surprisingly, even greater increases in bone turnover and a similar degree of bone loss were observed in ERαKO mice following castration. Moreover, T replacement completely prevented this ORX-induced bone loss, not only in WT mice, but also in ERαKO mice. The effects of T completely resemble the well-known antiresorptive actions of estradiol in ovariectomized mice and rats. This bone-sparing action of T in ERαKO mice is mediated by either the AR or ERβ. Again, the latter is unlikely, as mentioned earlier. These findings indicate that T exerts its bone-protective effect through nonERα-mediated pathways.

To study the extent to which the antiresorptive action of E2 in male mice is independent of ERα, we

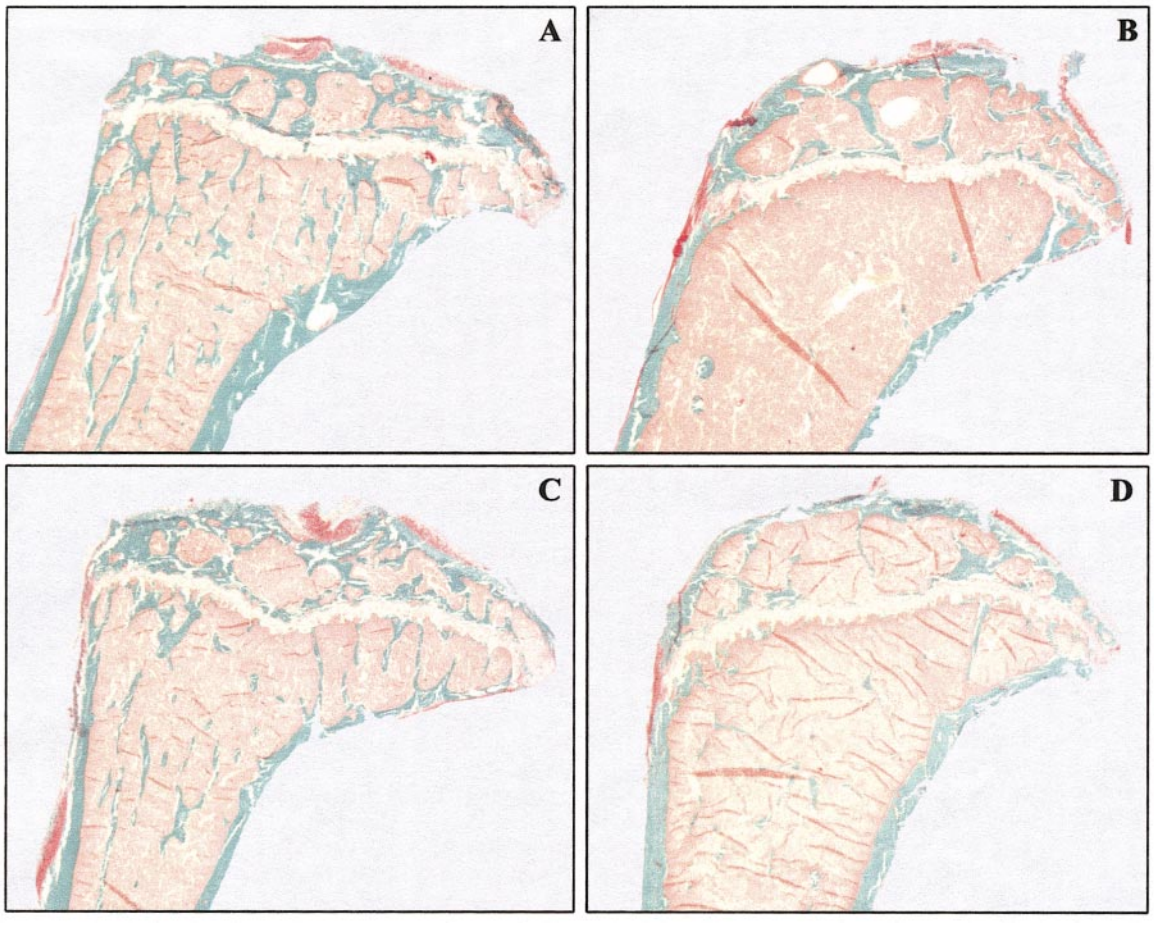

FIG. 4. Histological comparison of trabecular bone volume in the proximal tibial metaphysis in different experimental groups of male ERαKO mice. Longitudinal, undecalcified sections of the proximal tibia were stained by a modified Goldner technique (magnification 6×). (A) Sham ERαKO, (B) ORX ERαKO, (C) ORX+T ERαKO, (D) ORX+E2 ERαKO. Note the marked decrease of trabecular bone volume induced by orchidectomy in ERαKO mice. T prevents this ORX-induced bone loss, whereas ERαKO mice are resistant to E2 action.

evaluated the effects of E2 administration. E2 replacement clearly increased trabecular and cortical bone density in ORX WT mice. This effect was obtained by a remarkable stimulation of new bone formation: both osteoblast number and activity were significantly increased at the endocortical surface. The anabolic action of E2, which is very similar to the anabolic action of PTH, was already reported in female mice (27, 28), and was only recently described in male mice (29). Similar anabolic effects of E2 have been reported in postmenopausal women (30). Our results confirm that ERαKO mice are resistant to this anabolic action of E2, at least in the cancellous bone compartment. This suggests that the osteogenic response of high-dose estrogen is dependent on ERα activation. E2 did, however, significantly stimulate bone formation at the endocortical envelope, and to a minor extent at the periosteal envelope. This action of supraphysiological doses of E2 on cortical bone may be mediated by ERβ or via nongenotropic, sex-nonspecific signaling through the AR, as recently described by Kousteni *et al.* (31).

In conclusion, our findings confirm that male ERαKO mice have reduced longitudinal and radial bone growth, supporting the concept that ERα is necessary to obtain full skeletal size in male mice. ERα is, however, not important to maintain cancellous bone mass, since ERαKO mice have increased trabecular bone density. Furthermore, only T, and not E2, prevents orchidectomy-induced trabecular bone loss in ERαKO mice, suggesting that the bone-protective effect of T does not require ERα stimulation. Additionally, ERαKO mice are resistant to the anabolic action of high doses of estradiol in cancellous but not in cortical bone. With respect to sex steroid action, our study favours a role for ERβ activation in male cortical bone and AR stimulation in cancellous bone.

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