Estrogen receptor α, but not estrogen receptor β, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-κB ligand) ratio and serum interleukin-6 in male mice

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Abstract

Estrogens are important for the male skeleton. Osteoprotegerin (OPG), receptor activator of NF-κB ligand (RANKL), interleukin-6 (IL-6), IL-1 and tumor necrosis factor α (TNFα) have been suggested to be involved in the skeletal effects of estrogen. We treated orchidectomized mice with estradiol for 2 weeks and observed a 143% increase in the trabecular bone mineral density of the distal metaphysis of femur that was associated with a decreased OPG/RANKL mRNA ratio in vertebral bone. A similar decreased OPG/RANKL ratio was also seen after estrogen treatment of ovariectomized female mice. The effect of estrogen receptor (ER) inactivation on the OPG/RANKL ratio was dissected by using intact male mice lacking ERα (ERKO), ERβ (BERKO) or both receptors (DERKO). The expression of OPG was increased in ERKO and DERKO but not in BERKO male mice, resulting in an increased OPG/RANKL ratio. Furthermore, serum levels of IL-6 and tartrate-resistant acid phosphatase 5b (TRAP 5b) were decreased in ERKO and DERKO, but not in BERKO male mice. These results demonstrate that ERα, but not ERβ, is involved in the regulation of the vertebral OPG/RANKL ratio, serum levels of IL-6 and TRAP 5b in male mice.

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Introduction

Osteoporosis is a common disease, not only in women, but also in aging men. However, the mechanism behind male osteoporosis needs to be further studied. Androgens are known to be important for the male skeleton. The effect of androgens may be exerted either via the androgen receptor or via aromatization into estrogen and further via estrogen receptors (ERs). Orchidectomy has been shown to cause a decrease in bone mineral density (BMD) (Wakley *et al.* 1991, Gunness & Orwoll 1995) and an increase in bone resorption, which can be prevented by androgen treatment and also by treatment with the non-aromatizable androgen 5-dihydrotestosterone (Wakley *et al.* 1991). These results indicate that at least a part of the effect of androgens on the skeleton is mediated via the androgen receptor. However, there are increasing amounts of data indicating that estrogen is also of importance for the male skeleton. Inhibition of the enzyme aromatase results in a decrease in BMD (Vanderschueren *et al.* 1997). Patients with a mutation in the CYP19 gene, leading to aromatase deficiency, develop osteopenia (Morishima *et al.* 1995, Carani *et al.* 1997). Furthermore, recent clinical studies show a strong correlation between BMD and estrogen in males (Slemenda *et al.* 1997, Khosla *et al.* 1998, Gillberg *et al.* 1999). Thus, both clinical and experimental studies demonstrate that estrogen has an important role in the regulation of the male skeleton.

The effect of estrogen is mediated by binding to, and activation of, the ERs. These receptors are denoted as ERα and ERβ. We have recently shown that ERα, but not ERβ, is important for normal growth and maturation of the skeleton in male mice (Vidal *et al.* 2000).

The mechanism of action for estrogen to regulate adult skeletal metabolism is not yet fully understood. Several cytokines, including interleukin-1 (IL-1), tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and macrophage colony stimulating factor (M-CSF) have been suggested to

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be involved in the osteoprotective role of estrogen (Spelsberg *et al.* 1999, Cenci *et al.* 2000). Both ERα and ERβ are expressed by osteoblasts (Eriksen *et al.* 1988, Arts *et al.* 1997, Vidal *et al.* 1999). The effects of estrogen on osteoblasts include both direct effects on osteoblasts, resulting in increased bone formation, and indirect effects, via an osteoblast-mediated interaction with pre-osteoclasts and osteoclasts, resulting in a decreased bone resorption. Furthermore, ERs are also expressed by osteoclasts *in vivo* (Braidman *et al.* 2001). IL-6 has been shown to increase osteoclast formation (Tamura *et al.* 1993) and it has been suggested that down-regulation of IL-6 is important for mediating the protective effects of estrogen on the skeleton (Poli *et al.* 1994). Estrogen has been shown to decrease both the production of IL-6 (Kassem *et al.* 1996, Qu *et al.* 1999) and the IL-6 receptor (Lin *et al.* 1997), but there are also contradictory studies not showing any effect of estrogen on the levels of IL-6 (Chaudhary *et al.* 1992, Vargas *et al.* 1996). Another family of cytokines suggested to be involved in the effect of estrogen on the skeleton includes osteoprotegerin (OPG), receptor activator of NF-κB (RANK) and RANK ligand (RANKL) (Anderson *et al.* 1997, Simonet *et al.* 1997, Lacey *et al.* 1998). RANKL is a membrane-bound ligand expressed on the osteoblasts. Binding of RANKL to its receptor RANK, expressed on osteoclast precursors and mature osteoclasts, induces osteoclastogenesis and activation of mature osteoclasts. OPG prevents this interaction by binding to RANKL and thereby inhibits osteoclast formation and activation of mature osteoclasts. Transgenic mice overexpressing OPG suffer from osteopetrosis due to lack of osteoclasts while knockout mice devoid of OPG suffer from osteoporosis due to excessive osteoclastogenesis and activation of mature osteoclasts (Simonet *et al.* 1997, Mizuno *et al.* 1998). The relative abundance of OPG and RANKL (the OPG/RANKL ratio) has been suggested to be important in the regulation of osteoclastogenesis and activation of mature osteoclasts (Horwood *et al.* 1998). Estrogen has been shown to suppress RANKL-induced osteoclast differentiation *in vitro* (Shevde *et al.* 2000). Previous *in vitro* studies on the effect of estrogen on OPG expression have been contradictory. One study showed increased, and another study unchanged OPG expression after estrogen treatment (Vidal *et al.* 1998, Hofbauer *et al.* 1999). It is unknown if estrogen regulates the expression of OPG and/or RANKL *in vivo*.

The aim of the present study was to investigate the effects of estrogen deficiency and selective loss of ER expression on the OPG/RANKL ratio and serum levels of IL-6 in male mice.

Materials and Methods

Animals

Experiment 1, orchidectomized mice Mice, with a mixed C57BL/6J/129 background, were orchidectomized at 15 weeks of age. After rest for 1 week after castration, the mice were injected s.c. with 2·3 μg/mouse/day of 17β-estradiol benzoate (E_2), for 2 weeks. Control mice received injections of vehicle oil (Apoteksbolaget, Gothenburg, Sweden). Serum levels of 17β-estradiol were 52 ± 16 pg/ml in 17β-estradiol-treated orchidectomized mice, not detectable in vehicle-treated orchidectomized mice and the physiological level in intact male mice is 15 ± 0·5 pg/ml. At the end of the experiment, femurs and tibiae were excised and kept in 70% (v/v) ethanol for analysis by dual X-ray absorptiometry (DXA) and peripheral quantitative computerized tomography (pQCT). For histomorphometric studies, 26-week-old male mice, with a mixed C57BL/6J/129 background, were orchidectomized and treated with 0·7 μg/mouse/day of 17β-estradiol or vehicle oil, for 3 weeks.

Experiment 2, ovariectomized mice Three-month-old female mice were ovariectomized and treated with 2·3 μg/mouse/day of 17β-estradiol or vehicle oil, for 3 weeks. Serum levels of 17β-estradiol were 112 ± 45 pg/ml in 17β-estradiol-treated ovariectomized mice, not detectable in vehicle-treated ovariectomized mice and the physiological level in intact female mice is 33 ± 1·1 pg/ml. At sacrifice the vertebrae were dissected and used for RT-PCR analysis of OPG and RANKL.

Experiment 3, intact ER inactivated male mice Male double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated with female double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice, resulting in wild type (WT), $ER\alpha^{-/-}\beta^{+/+}$ (ERKO), $ER\alpha^{+/+}\beta^{-/-}$ (BERKO) and $ER\alpha^{-/-}\beta^{-/-}$ (DERKO) offspring, with a mixed C57BL/6J/129 background. Genotyping was performed as previously described (Lubahn *et al.* 1993, Krege *et al.* 1998). Animals had free access to fresh water and food pellets (B&K Universal AB, Sollentuna, Sweden) consisting of cereal products (76·9% barley, wheat feed, wheat and maize germ), vegetable proteins (14·0% hipro soya) and vegetable oil (0·8% soya oil).

Dual X-ray absorptiometry (DXA)

The areal bone mineral density (areal BMD) and bone mineral content (BMC) were measured with the Norland pDEXA Sabre (Fort Atkinson, WI, USA) and analyzed using the Sabre Research software (3·6) as previously described (Windahl *et al.* 1999). The distal end of the femur contains a relatively high proportion of trabecular bone and a scan (4 × 4 mm) at this site of the femur was performed to estimate the effects of estrogen on trabecular BMD with the DXA technique. The inter-assay coefficient of variation was less than 5% (Andersson *et al.* 2001).

Peripheral quantitative computerized tomography (pQCT)

The trabecular BMD was analyzed by a metaphyseal scan of the distal femur using the Stratec pQCT XCT

Research M (Norland, software version 5·4B) with a resolution of 70 μm as previously described (Windahl *et al.* 1999). The inter-assay coefficient of variation was less than 2% (Vidal *et al.* 2000).

Histomorphometry

The left tibia was fixed in 10% phosphate-buffered formalin, embedded in methacrylate resin, sectioned and stained by Goldner's Trichrome method. Analysis of trabecular bone was restricted to an area 0·25–2 mm in a diaphyseal direction from the growth plate, maintaining separation between the analysis area and the cortical wall. Bone histomorphometric analysis was carried out using an Osteomeasure histomorphometry workstation incorporating a Nikon E400 microscope with Plan Fluor objectives, and Osteomeasure histomorphometry software. Histomorphometric parameters included: trabecular bone volume (BV/TV; %), osteoid volume (OV/BV; %), osteoid surface (OS/BS; %), trabecular thickness (Tb.Th; μm), trabecular separation (Tb.Sp; μm), trabecular number (Tb.N; mm^{-1}), eroded surface (ES/BS; %) and osteoclast number (N.Oc/B.Pm; mm^{-1}) (Parfitt *et al.* 1987).

Probes

A cDNA fragment of 262 bp corresponding to parts of the full-length mouse OPG cDNA (gbMMU94331) and a cDNA fragment of 399 bp corresponding to parts of the full-length mouse RANKL cDNA (gbAF053713) were generated with RT-PCR. For OPG, total RNA from murine liver and the following primer pairs were used: 5′-GTG AGG AAG GGC GTT ACC-3′ and 5′-TTT TGC GTG GCT TCT CTG-3′. For RANKL, total RNA from murine spleen and the following primer pairs were used: 5′-ATC GGG TTC CCA TAA AGT-3′ and 5′-CCA AAG TAC GTC GCA TCT-3′. The OPG and RANKL PCR products were inserted into a Bluescript vector and pCRII vector respectively by T/A cloning, and the sequences were verified by sequencing. The vectors were made linear with SpeI (OPG) and EcoRV (RANKL) prior to *in vitro* transcription with T3-polymerase (OPG) or SP6-polymerase (RANKL) in the presence of [^{32}P]α-UTP. The ribosomal 18S (Ambion, Austin, TX, USA) was used as an internal standard. The 18S vector was made linear with HindIII and T7-polymerase for *in vitro* transcription and incorporation of [^{33}P]α-UTP.

RNAse protection assay

The OPG and RANKL mRNA levels were measured using RNAse protection assay in experiments 1 and 3. RNA was prepared as described elsewhere (Chomczynski & Sacchi 1987). Due to technical reasons, the trabecular BMD was measured in the distal femur while the RNA was prepared from vertebrae and one cannot exclude that different skeletal sites respond differentially to estrogen treatment. Quantification of the OPG and RANKL transcripts was performed using RNAse protection assay (RPA kit II and III, Ambion). Thirty micrograms of total RNA from vertebrae was used for hybridization and the RNA–RNA hybrids were separated by polyacrylamide gel electrophoresis. Visualization of the bands was performed using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA, USA). Quantification of the bands was performed in ImageQuant (Molecular Dynamics, Version 3·3). The intensity of the OPG band, related to 18S, was divided by the intensity of the RANKL band, related to 18S, to calculate the OPG/RANKL ratio.

cDNA synthesis

cDNA was generated from 1 μg total RNA from vertebrae in reverse transcription buffer with avian myeloblastosis virus (AMV) RT, dNTP, random primers and RNase inhibitor (Promega, Madison, WI, USA). The reaction conditions were 5 min at 22 °C, 50 min at 42 °C and 5 min at 72 °C.

Real-time PCR

The OPG and RANKL mRNA levels were measured using real-time PCR in experiment 2. The sequence for the forward primer for RANKL (accession no. AB022036S) was 5′-GCA CAC CTC ACC ATC AAT GCT-3′ corresponding to nucleotides 336–356 in exon 4 and for the reverse primer 5′-GGT ACC AAG AGG ACA GAG TGA CTT TA-3′ corresponding to nucleotides 182–168 in exon 5. The sequence of the TaqMan probe was 5′-CCA GCA TCC CAT CGG GTT CCC-3′ corresponding to nucleotides 358 in exon 4 to 164 in exon 5. The sequence for the forward primer for OPG (accession no. AB013899S1) was 5′-TGA GTG TGA GGA AGG GCG TTA-3′ corresponding to nucleotides 290–310 in exon 2 and for the reverse primer 5′-CCA TCT GGA CAT TTT TTG CAA A-3′ corresponding to nucleotides 51–30 in exon 3. The sequence of the TaqMan probe was 5′-AGC ACC GGA GCT GTC CCC CG-3′ corresponding to nucleotides 334–353 in exon 2.

The oligonucleotide primers and probe for 18S rRNA were purchased from PE Applied Biosystems (Stockholm, Sweden). All probes apart from 18S rRNA, which was labeled with VIC and TAMRA, were fluorescein labeled with the reporter dye FAM and quencher dye TAMRA. The cDNA from four animals was pooled and run in triplicate. The analysis was repeated three times. The cDNA was amplified using ABI PRISM 7700 (PE Applied Biosystems, Stockholm, Sweden) at the following conditions: one cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C

for 1 min. The mRNA amount of each gene was calculated using the 'Standard Curve Method' (separate tubes, following the instructions in User Bulletin no. 2, PE Applied Biosystems) and adjusted for the expression of 18S rRNA.

IL-6 bioassay

Levels of IL-6 in serum were measured by a sensitive bioassay as previously described (Bremell *et al.* 1992). Briefly, the sub clone B9 from the cell line B13·29, which is dependent on IL-6 for growth, was seeded into microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 5000 cells per well. The cells were cultured in complete Iscove's medium with serum samples for 72 h. After 68 h of culture [^{3}H]thymidine was added to each well. The samples were tested at two different dilutions and the radioactive incorporation was compared with that of a recombinant mouse IL-6 standard.

TRAP 5b immunoassay

Tartrate-resistant acid phosphatase (TRAP) 5b was purified from human osteoclasts as described (Halleen *et al.* 1996), and the purified enzyme was used as antigen to develop a polyclonal antiserum in rabbits (Alatalo *et al.* 2000). The antiserum was incubated on antirabbit IgG-coated microtiter plates (EG & G Wallac, Turku, Finland) for 1 h. Diluted mouse serum samples were incubated in the wells for 1 h, and bound enzyme activity was detected using 8 mmol/l 4-nitrophenyl phosphate (4-NPP) as substrate in 0·1 mol/l sodium acetate buffer pH 6·1 for 2 h at 37 °C. The enzyme reactions were terminated by adding 25 µl of 0·32 mol/l NaOH to the wells, and A405 was measured using Victor^2 equipment (EG & G Wallac).

17β-estradiol RIA

17β-estradiol was measured using a RIA detecting estradiol (DiaSorin, Saluggia, Italy), with a sensitivity of 10 pg/ml.

Results

Experiment 1

Estrogen increases trabecular bone mineral density in orchidectomized mice To determine the effect of estrogen on the trabecular BMD, wild type mice were orchidectomized at 15 weeks of age and thereafter given estrogen or vehicle for 2 weeks. Treatment with estrogen did neither regulate the body weight nor the lengths of femur or tibia (data not shown). DXA measurements of the distal end of femur, which contains a relatively high proportion of trabecular bone, demonstrated that estrogen increased the areal BMD and BMC (BMD: +27%, BMC: +38%, Table 1). Furthermore, treatment with estrogen resulted in a significantly increased trabecular volumetric BMD in male mice, as measured in a metaphyseal scan of the distal femur using pQCT (+143%, Fig. 1A,B).

Table 1 DXA measurements of distal femur. Areal BMD and BMC measurements using DXA on the distal part of femur (4 × 4 mm) from orchidectomized mice treated with vehicle (V) or 17β-estradiol (E) for 2 weeks. Values are given as means ± S.E.M. (vehicle $n=4$, estrogen $n=4$)

	V	E
Areal BMD (mg/cm^2)	61·1 ± 5·1	77·7 ± 3·7*
BMC (mg)	7·9 ± 0·6	10·8 ± 0·6*

*$P<0·05$ vs vehicle, Student's *t*-test.

Effect of estrogen treatment on histomorphometric indices in orchidectomized mice Trabecular bone volume (BV/TV) was increased after estrogen treatment of orchidectomized mice, confirming the increased trabecular BMD as measured by pQCT (Table 2). The increase in trabecular bone volume was associated with an increase in trabecular number (Tb.N.) and thickness (Tb.Th.; Table 2). Osteoid volume (OV/BV), eroded surface (ES/BS) and the number of osteoclasts (N.Oc./B.Pm.) were decreased in the estrogen-treated orchidectomized mice, indicating that estrogen treatment resulted in a decreased bone turnover (Table 2).

Estrogen decreases the OPG/RANKL ratio in orchidectomized male mice We next wanted to study if the estrogen-induced increase in trabecular BMD was associated with an altered expression of OPG and RANKL. The mRNA expression of OPG and RANKL was measured on total RNA isolated from vertebrae using RNase protection assay. The OPG/RANKL ratio was decreased by 41% after 2 weeks of treatment with estrogen (Fig. 1C).

Experiment 2

Estrogen decreases the OPG/RANKL ratio in ovariectomized female mice The mRNA expression of OPG and RANKL was also analyzed in ovariectomized female mice treated either with estrogen or the vehicle using real-time PCR. There was a tendency to a decrease in the expression of OPG (–36 ± 6%), while the expression of RANKL was increased (133 ± 9%, $P<0·01$, Student's *t*-test) in 17β-estradiol-treated mice compared with vehicle-treated mice. This resulted in a decrease in

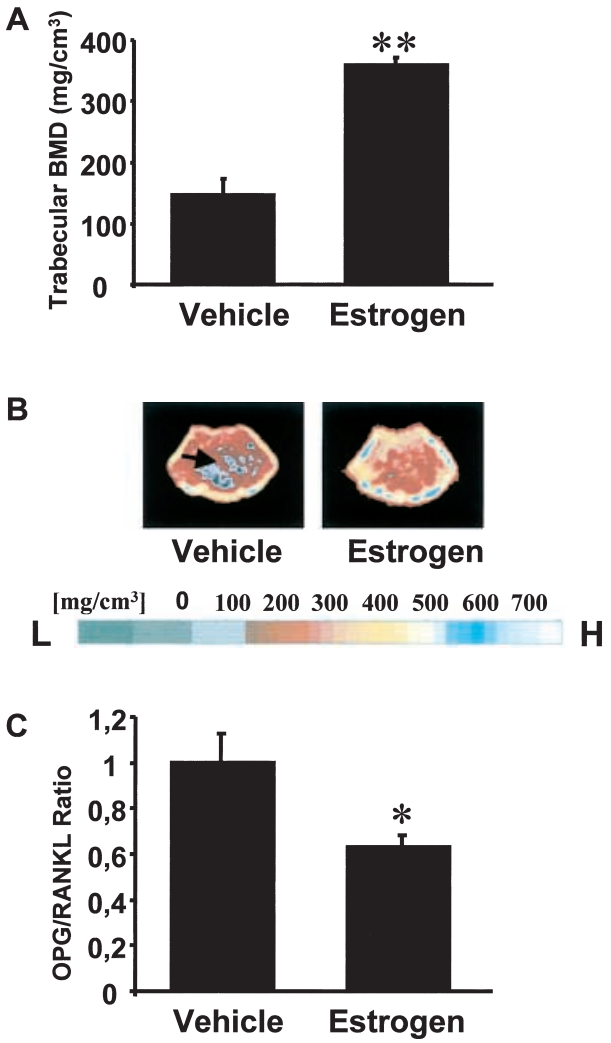

Figure 1 (A) Effect of estrogen on trabecular BMD in orchidectomized male wild type mice as determined by a metaphyseal pQCT scan of the distal femur. Values are given as means ± S.E.M. (vehicle $n=4$, estrogen $n=4$). **$P<0{\cdot}01$ estrogen versus vehicle, Student's t-test. (B) Representative metaphyseal scan of vehicle-treated and estrogen-treated orchidectomized male mice. Arrow indicates a central area consisting of trabecular bone. (C) Effect of estrogen on the ratio of the mRNA expression of OPG to the mRNA expression of RANKL in vertebrae in orchidectomized male mice, as measured using RNase protection assay. Values are given as means ± S.E.M. (vehicle $n=4$, estrogen $n=3$). *$P<0{\cdot}05$, Student's t-test.

the OPG/RANKL ratio (–73 ± 2%, $P<0{\cdot}05$, Student's t-test), similar to what was seen after estrogen treatment of orchidectomized male mice.

Experiment 3

ERα but not ERβ is involved in the regulation of the OPG/RANKL ratio in male mice The effect of ER inactivation on the OPG/RANKL ratio, as measured using RNase protection assay, was investigated by using mice lacking ERα (ERKO), ERβ (BERKO) or both receptors (DERKO). The OPG/RANKL ratio was unchanged when comparing WT and BERKO males. However, in ERKO and DERKO males, this ratio was increased by 69% and 70% respectively (Fig. 2A). The altered OPG/RANKL ratio was associated with an increase in OPG mRNA expression (36% in both ERKO and DERKO) (Fig. 2B,C).

Table 2 Effect of estrogen treatment on histomorphometric indices in orchidectomized mice. Histomorphometric indices were measured in the metaphyseal region of the proximal tibiae in orchidectomized mice treated with either vehicle (V) or 17β-estradiol (E) for 3 weeks ($n=5$–6). Values are given as means ± S.E.M.

	V	E
BV/TV (%)	3·0 ± 0·8	11·7 ± 3·3*
OV/BV (%)	8·6 ± 1·5	4·2 ± 0·2*
BS/BV (mm^{-1})	79 ± 10	46 ± 3*
Tb.Th. (μm)	27 ± 3	44 ± 3**
Tb.Sp. (μm)	983 ± 206	419 ± 106*
Tb.N. (mm^{-1})	1·1 ± 0·2	2·6 ± 0·7*
ES/BS (%)	13·1 ± 1·5	6·1 ± 1·3*
N.Oc./B.Pm. (mm^{-1})	11·6 ± 2·2	2·7 ± 0·8**

*$P<0{\cdot}05$, **$P<0{\cdot}01$ vs vehicle, Student's t-test. BV/TV=bone volume/total volume, OV/BV=osteoid volume/bone volume, BS/BV=bone surface/bone volume, Tb.Th.=trabecular thickness, Tb.Sp.=trabecular separation, Tb.N.=trabecular number, ES/BS=eroded surface/bone surface, N.Oc./B.Pm.=number of osteoclasts.

Altered serum TRAP 5b levels in male mice lacking ERα Since a high OPG/RANKL ratio suggests low bone turnover, we measured the serum levels of TRAP 5b, which is a specific marker of bone resorption (Halleen *et al.* 2000). Serum TRAP 5b levels were decreased in both ERKO (–59%) and DERKO (–54%), while they were unchanged in BERKO males, indicating a low bone turnover in male mice lacking ERα (Fig. 3A).

ERα but not ERβ is involved in the regulation of serum IL-6 levels Measurement of serum IL-6 levels using a sensitive bioassay revealed a significant reduction in the IL-6 levels in ERKO (–68%) and in DERKO males (–59%), while the levels in BERKO males were unchanged compared with WT (Fig. 3B).

Histomorphometric indices in ER inactivated mice We have previously published data that the trabecular bone mineral density, as measured using pQCT, as well as the trabecular bone volume (BV/TV), as measured using histomorphometric analysis, are unchanged in male ER inactivated mice (Vidal *et al.* 2000, Table 3). Histomorphometric analysis, in the present study, revealed that $ER\alpha^{-/-}$ but not $ER\beta^{-/-}$ mice display a reduced trabecular

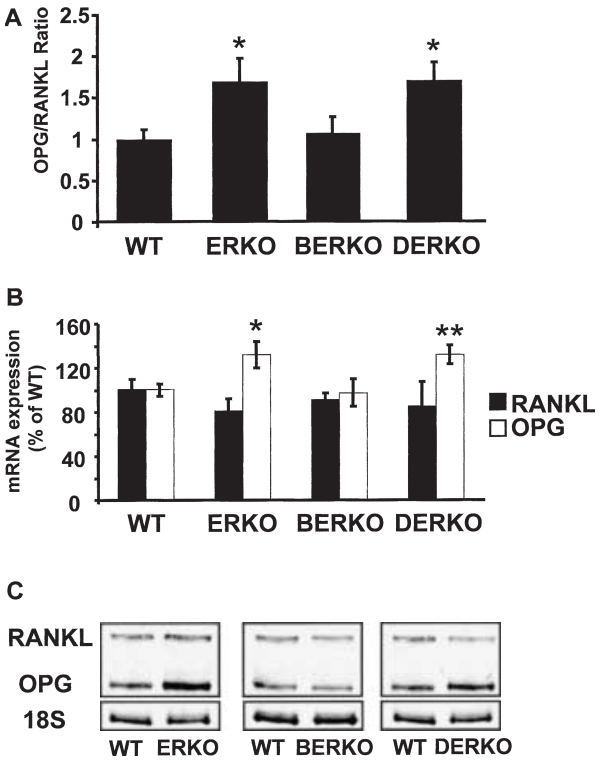

Figure 2 Estrogen receptor specificity for the regulation of vertebral OPG and RANKL mRNA expression in male mice. The OPG/RANKL ratio (A) and the OPG and RANKL mRNA levels (B) were measured in male wild type (WT), ERKO, BERKO and DERKO mice using RNase protection assay. Representative RNase protection assays are shown in (C). Values are given as means ± S.E.M. (n=5–6). *P<0·05, **P<0·01 versus WT, Student's t-test.

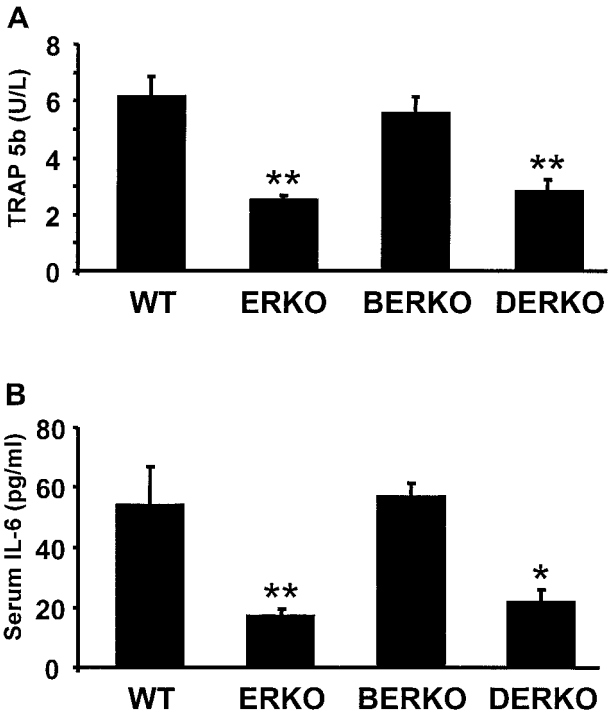

Figure 3 Estrogen receptor specificity for the regulation of serum TRAP 5b (A) and IL-6 (B) levels in male wild type (WT), ERKO, BERKO and DERKO mice. Values are given as means ± S.E.M. (n=5–6). *P<0·05, **P<0·01 versus WT, Student's t-test.

thickness (Tb.Th.) and increased number of trabeculae (Tb.N.), resulting in an increased bone surface/bone volume (BS/BV, Table 3). However, these effects were small and further experiments might be needed before any clear conclusions regarding the trabecular bone microarchitecture in male ER$\alpha^{-/-}$ mice could be drawn. The osteoid volume (OV/BV), eroded surface (ES/BS) and the number of osteoclasts (N.Oc/B.Pm) were unchanged in all genotypes (Table 3).

Discussion

Androgens are well known to be important for the male skeleton, but recent studies indicate that estrogen also plays a role in the regulation of this tissue. We have previously demonstrated that estrogen is of importance for skeletal growth and maturation in male mice (Vidal *et al.* 2000). In the present study we have shown that estrogen increases the trabecular BMD in orchidectomized adult mice, demonstrating that estrogen is important for the adult male skeleton as well. This increase in trabecular BMD was associated with a decrease in the OPG/RANKL ratio. Furthermore, the stimulatory effect of estrogen on the OPG/RANKL ratio in gonadectomized mice was not specific for male mice as in an additional experiment we found similar results in female mice. An inhibitory effect of estrogen on OPG expression is supported by a study describing increased serum levels of OPG in postmenopausal women (Yano *et al.* 1999). In contrast, an *in vitro* study showed that estrogen increases the expression of OPG (Hofbauer *et al.* 1999). Since our data are *in vivo* data where estrogen is given to gonadectomized mice, it is difficult to directly compare them with the previous *in vitro* and clinical studies where estrogen regulates OPG and RANKL expression. Histomorphometric analysis of orchidectomized mice indicated that the bone turnover was decreased in estrogen-treated mice. Thus, the observed decreased OPG/RANKL ratio in these mice might be a result of a secondary feedback mechanism with the aim to increase the bone turnover to normal levels rather than a direct effect of estrogen on the OPG/RANKL ratio.

The ER specificity for the regulation of the OPG/RANKL ratio was studied in intact mice devoid of one or both of the known ERs. Loss of both ERs resulted in an increase in the OPG/RANKL ratio and this increase was

Table 3 Histomorphometric indices in ER inactivated mice. Histomorphometric indices were measured in the metaphyseal region of the proximal tibiae in mice lacking ERα (ERKO), ERβ (BERKO) or both ERs (DERKO) (n=5–6). Values are given as means ± S.E.M.

	WT	ERKO	BERKO	DERKO	$ERα^{-/-}$	$ERβ^{-/-}$
BV/TV (%)	14·3 ± 1·2	13·3 ± 0·5	17·8 ± 3·9	13·9 ± 1·7	NS	NS
OV/BV (%)	0·63 ± 0·26	0·59 ± 0·43	0·57 ± 0·12	0·50 ± 0·13	NS	NS
BS/BV (mm^{-1})	68·1 ± 3·0	83·4 ± 2·1	62·2 ± 7·9	91·9 ± 9·5	P<0·01	NS
Tb.Th. (μm)	29·6 ± 1·3	24·1 ± 0·6	35·4 ± 5·4	22·4 ± 2·0	P<0·01	NS
Tb.Sp. (μm)	182 ± 16	158 ± 5	188 ± 32	142 ± 10	NS	NS
Tb.N. (mm^{-1})	4·8 ± 0·4	5·5 ± 0·1	4·8 ± 0·5	6·1 ± 0·3	P<0·05	NS
ES/BS (%)	9·0 ± 4·1	6·9 ± 1·5	10·0 ± 1·7	5·6 ± 1·7	NS	NS
N.Oc./B.Pm. (mm^{-1})	9·0 ± 4·0	6·0 ± 1·1	5·0 ± 1·8	6·6 ± 2·4	NS	NS

A two-way ANOVA followed by Student–Neuman–Keul's multiple range test was performed in which $ERα^{-/-}$ and $ERβ^{-/-}$ were regarded as separate treatments. The P-value versus the respective +/+ allele is indicated. NS=non significant. BV/TV=bone volume/total volume, OV/BV=osteoid volume/bone volume, BS/BV=bone surface/bone volume, Tb.Th.=trabecular thickness, Tb.Sp.=trabecular separation, Tb.N.=trabecular number, ES/BS=eroded surface/bone surface, N.Oc./B.Pm.=number of osteoclasts.

also observed in the ERKO, but not in the BERKO males, demonstrating that ERα, but not ERβ, is involved in the maintenance of a normal ratio between OPG and RANKL. The ERKO and DERKO males do not suffer from trabecular osteopenia, but the cortical BMC is decreased because the bones are smaller in size (Vidal *et al.* 2000, Sims *et al.* 2000). ERKO and DERKO mice have reduced serum levels of insulin-like growth factor-I (IGF-I) and osteocalcin (Vidal *et al.* 2000), indicating that male ERKO and DERKO mice have a low bone turnover. This notion is further supported by the decrease in serum TRAP 5b levels found in these animals. TRAP 5b is an osteoclast-specific enzyme that is secreted into the circulation. Serum TRAP 5b levels increase after menopause and decrease during estrogen replacement therapy (Halleen *et al.* 2000), suggesting that serum TRAP 5b is a useful marker to monitor estrogen-induced changes in bone resorption.

IL-6 stimulates osteoclast formation (Tamura *et al.* 1993) and has been suggested to be an important mediator of the negative effects of estrogen deficiency on bone. Estrogen has been shown to decrease the production of IL-6 (Girasole *et al.* 1992, Qu *et al.* 1999) and estrogen withdrawal has been shown to result in an increased production of IL-6 (Passeri *et al.* 1993) *in vitro*. Other studies showed no effects of estrogen on IL-6 production (Chaudhary *et al.* 1992, Vargas *et al.* 1996). In our study we found that loss of both ERs resulted in a decrease in the IL-6 serum levels. Similarly to what was seen for the OPG/RANKL ratio, this effect on serum IL-6 levels was dependent on ERα but not on ERβ. It has recently been shown that aromatase-deficient male mice have a decreased bone turnover (Oz *et al.* 2000). In similarity, some of our data indicate that loss of ERα results in low bone turnover, suggesting that estrogen acts via ERα to regulate bone turnover in male mice. The importance of ERα in the regulation of male skeletal metabolism is supported by a recent study demonstrating impaired ERα protein expression in men with idiopathic osteoporosis (Braidman *et al.* 2000).

In conclusion, estrogen increases trabecular BMD, and this increase is associated with a decreased OPG/RANKL ratio in both male and female mice. ERα, but not ERβ, is involved in the regulation of the skeletal OPG/RANKL ratio and the regulation of serum levels of IL-6 and TRAP 5b in male mice.

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