Estrogen receptor specificity for the effects of estrogen in ovariectomized mice

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Abstract

Estrogen exerts a variety of important physiological effects, which have been suggested to be mediated via the two known estrogen receptors (ERs), α and β . Three-month-old ovariectomized mice, lacking one or both of the two estrogen receptors, were given estrogen subcutaneously (2.3 µg/mouse per day) and the effects on different estrogen-responsive parameters, including skeletal effects, were studied. We found that estrogen increased the cortical bone dimensions in both wild-type (WT) and double ER knockout (DERKO) mice. DNA microarray

analysis was performed to characterize this effect on cortical bone and it identified four genes that were regulated by estrogen in both WT and DERKO mice. The effect of estrogen on cortical bone in DERKO mice might either be due to remaining ER α activity or represent an ER α /ER β -independent effect. Other effects of estrogen, such as increased trabecular bone mineral density, thymic atrophy, fat reduction and increased uterine weight, were mainly ER α mediated.

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Introduction

Estrogen is the most frequently prescribed medication in the United States. Estrogen deficiency increases the risk of a wide variety of illnesses, including cardiovascular diseases, obesity, rheumatoid arthritis, osteoporosis and neuro-degenerative diseases, while estrogen substitution reduces the risk of these ailments (Cosman & Lindsay 1999). However, long-term estrogen replacement therapy is also associated with side-effects including increased risk of breast cancer and deep venous thrombosis (Daly *et al.* 1996, Collaborative Group on Hormonal Factors in Breast Cancer 1997). Agents that can maintain the benefit of estrogen but avoid the risks are therefore needed. Most of the effects of estrogen are exerted via the two known estrogen receptors (ERs), ER α and ER β . An increased understanding of the receptor specificity in different organs for the effects of estrogen is of importance for the development of such agents. We have, in the present study, determined ER specificity for the effects of estrogen on adult bone metabolism, the immune system, fat mass and the uterus.

Previous experimental bone studies have demonstrated that ovariectomy reduces trabecular bone mineral density (BMD) as well as the cross-sectional cortical bone area while estrogen substitution of ovariectomized (ovx) mice restores both these bone compartments (Turner 1999, Windahl *et al.* 1999, Daci *et al.* 2000). Estrogens are also known to exert multiple effects on the development and regulation of the immune system (Olsen & Kovacs 1996). It is well established that estrogen is important for the development of the thymus and for estrogen-induced thymic atrophy during pregnancy (Clarke & Kendall 1994). Exposure of adult mice to endogenous or exogenous estrogen induces massive reduction of thymic weight and cellularity (Screpanti *et al.* 1989).

It is well known that ovariectomy results in increased fat stores and body weight in female rodents (Couse & Korach 1999). This effect can be prevented with estrogen and reproduced with anti-estrogen treatment in intact females (Couse & Korach 1999). A fat-reducing effect of endogenous estrogen is supported by the fact that aromatase-inactivated as well as ER α -inactivated mice are obese (Heine *et al.* 2000, Jones *et al.* 2000, Ohlsson *et al.* 2000).

A definitive role for ER α in the uterotrophic effects of estrogen has been confirmed in adult female ER α knock-out mice, where there is loss of estrogen responsiveness (Lubahn *et al.* 1993), as well as in mice with disruption of the estrogen-responsive ring finger protein gene (Orimo *et al.* 1999). ER β is present in both endometrium and myometrium and we have reported that ER β inactivated (BERKO) mice display an exaggerated estrogenic response in the immature uterus (Weihua *et al.* 2000). The aim of the present study was to determine the ER specificity for several different effects of estrogen in ovx mice.

Materials and Methods

Animals

Male double heterozygous (ER $\alpha^{+/-}\beta^{+/-}$) mice were mated with female double heterozygous (ER $\alpha^{+/-}\beta^{+/-}$) mice, resulting in ER $\alpha^{+/+}\beta^{+/+}$ =wild-type (WT), ER $\alpha^{-/-}\beta^{+/+}$ =ERKO, ER $\alpha^{+/+}\beta^{-/-}$ =BERKO and ER $\alpha^{-/-}\beta^{-/-}$ =DERKO offsprings (Lubahn *et al.* 1993, Kregge *et al.* 1998, Vidal *et al.* 2000). The diet, housing and genetic background were as previously described (Vidal *et al.* 2000). In the estrogen-exposure experiments, all mice were ovariectomized at 2 months of age. Ovaries were removed after a flank incision and the incisions were closed with metallic clips. After recovery for 4 days, mice were injected s.c with 17 β -estradiol benzoate (2.3 μ g/mouse per day; Sigma, St Louis, MO, USA) for 5 days/week for 3 weeks. Control mice received injections of vehicle oil (olive oil; Apoteksbolaget, Göteborg, Sweden). The dose of 17 β -estradiol chosen resulted in a serum concentration of 514 ± 184 pM. Normal serum levels of estradiol are between 70 and 110 pM in diestrus while they are between 350 and 730 pM in estrus (Offner *et al.* 2000). Thus, our 17 β -estradiol treatment resulted in serum levels that were similar to those normally seen during estrus in female mice. The results on cortical bone parameters were also seen in an additional large experiment ($n \geq 10$ in each group) in which ovx WT and DERKO mice were treated with placebo pellets or estradiol-containing pellets (Innovation Research, Sarasota, FL, USA) to re-establish circulating physiological levels of estradiol as previously described (Iafrati *et al.* 1997).

Peripheral quantitative computerized tomography (pQCT)

Computerized tomography was performed with the Stratec pQCT XCT Research M (software version 5.4B; Norland, Fort Atkinson, WI, USA) operating at a resolution of 70 μ m as previously described (Windahl *et al.* 1999, Vidal *et al.* 2000). Mid-diaphyseal pQCT scans of femur were performed to determine the cortical bone

mineral content (BMC), cortical cross-sectional area and cortical thickness. The mid-diaphyseal region of the femur and humerus in mice contains mostly cortical bone. Metaphyseal pQCT scans of distal femur and proximal humerus were performed to measure trabecular volumetric BMD. The trabecular bone region was defined by setting an inner threshold to 45% of the total area.

Dual X-ray absorptiometry (DXA)

DXA measurement was performed with the Norland pDEXA Sabre (Norland) and the Sabre Research software (version 3.6) as previously described (Windahl *et al.* 1999).

Western immunoblotting

Western immunoblotting was performed as previously described (Skrtic *et al.* 1997), with some modifications. Protein (50 μ g), prepared from frozen bone or liver tissue, was subjected to 4–12% Bis-Tris (Novex, San Diego, CA, USA) gel electrophoresis. The primary antibody (ER α , H-184; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:200 and the horseradish peroxidase-conjugated secondary antibody was diluted 1:2500.

DNA microarray analysis

RNA from the humerus was prepared as described elsewhere (Chomczynski & Sacchi 1987). The RNA was further purified using RNeasy Kit (Qiagen, Chatsworth, CA, USA). RNA from six different mice was prepared from each animal group (vehicle-treated WT, estrogen-treated WT, vehicle-treated DERKO and estrogen-treated DERKO). For microarray analysis, the six RNA samples were pooled in two groups of three each, resulting in two pools per animal group. The pooled RNA was reverse transcribed into cDNA, labeled and analysed by DNA microarray (MG-U74A Array; Affymetrix, Santa Clara, CA, USA). The array represents approximately 6000 mouse genes and approximately 3000 uncharacterized expression sequence tag (EST) clones. Preparation of labeled cRNA and hybridization was done according to the Affymetrix Gene Chip Expression Analysis manual.

Bioinformatics

Scanned output files were analysed using Affymetrix Micro Array Suite Version 4.0.1 software. To allow comparison of gene expression, the gene chips were globally scaled to an average intensity of 500. The estrogen-regulated genes were determined by calculating the fold change on average between vehicle-treated and estrogen-treated bone samples. For each genotype, comparisons were made between the two vehicle-treated and the two estrogen-treated gene chips, generating a total of four comparisons. An average fold change and

Table 1 Primers and probes used in the real-time PCR analysis

Bone sialoprotein			
Acc. no. L20232	Forward primer	(438–458)	5'-ACC CCA AGC ACA GAC TTT TGA-3'
Acc. no. L20232	Reverse primer	(519–500)	5'-CAC TTT TGG AGC CCT GCT TT-3'
Acc. no. L20232	Probe	(498–476)	5'-TGC ATC TCC AGC CTT CTT GGG CA-3'
MMP-9			
Acc. no. X72795	Forward primer	(1197–1219)	5'-ACA ATC CTT GCA ATG TGG ATG TT-3'
Acc. no. X72795	Reverse primer	(1284–1262)	5'-AAC TTC CAG TAC CAA CCG TCC TT-3'
Acc. no. X72795	Probe	(1252–1229)	5'-CAG AGC GCC CTG GAT CTC AGC AAT-3'
EST clone			
Acc. no. AI850558	Forward primer	(320–345)	5'-AAA TCA AGA CAT GGT TAT TGC TGA CT-3'
Acc. no. AI850558	Reverse primer	(393–372)	5'-CCC AAC AGT GAA AAT GCT TGA A-3'
Acc. no. AI850558	Probe	(347–368)	5'-CCG TCC GGC TCA CAT GCA CAG A-3'
Collagen VIII			
Acc. no. X66976	Forward primer	(250–274)	5'-AGT ACC CAT ACC TCC CCC AAT ATA T-3'
Acc. no. X66977	Reverse primer	(85–66)	5'-AAC TGG CTA ACG GTA CTT CTT TGC-3'
Acc. no. X66976	Probe	(276–302)	5'-AAG GAA ATC CCA CCT GTG CCA AGA ATG-3'

MMP-9, matrix metalloproteinase 9.

coefficient of variation (CV) for the four comparisons were calculated. This calculated CV was dependent both on the assay variation and on the biological variation between the two pools included in each experimental group. We defined very strict criteria for genes to be regarded as regulated in DERKO mice: (1) the absolute call for the gene had to be present (Affymetrix Micro Array Suite Version 4.0.1) for all gene chips, (2) the average fold increase or decrease of the four comparisons should be at least 2.5-fold and (3) the CV for these four comparisons should be less than 30%. Thus, we probably excluded some estrogen-regulated genes but this was done in order to avoid false positively regulated genes.

Real-time PCR analysis

The sequences for primers and probes that were used are described in Table 1. The analyses were performed using probes labeled with the reporter fluorescent dye FAM. Pre-designed primers and a probe labeled with the reporter fluorescent dye VIC, specific for 18S rRNA, were included in the reactions as an internal standard. All oligonucleotide primers and probes were purchased from PE Applied Biosystems (Stockholm, Sweden). The cDNA was amplified using ABI PRISM 7700 (PE Applied Biosystems) under 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' (multiplex reactions, following the instructions in User Bulletin No. 2, PE Applied Biosystems) and adjusted for the expression of 18S rRNA.

Thymus cellularity

Thymus glands were removed and weighed. Single cell suspensions were obtained after tissue was mashed and

passed through a nylon wool sieve. The cells were centrifuged at 515 *g* for 5 min and pelleted cells were resuspended in phosphate-buffered saline and the total number of thymocytes was calculated, using an automated cell counter (Sysmex, Kobe, Japan).

Sucrose gradient analysis

Frozen tissues (200 mg liver and 50 mg uterus) were pulverized in a dismembrator (Braun, Melsungen, Germany) for 45 s at 1800 r.p.m. Pulverized tissue was added to a buffer composed of 10 mM Tris-HCl, pH 7.5, 1.5 mM EDTA and 5 mM sodium molybdate. Cytosols were obtained by centrifugation of the homogenates for 1 h at 4 °C at 204 000 *g* in a 70 Ti rotor. Cytosols were incubated for 3 h at 0 °C with 10 nM 6,7-³H-labeled E₂ (48 Ci/mmol; New England Nuclear, Boston, MA, USA), and the bound and unbound steroids were separated with dextran-coated charcoal. Sucrose gradient sedimentation was then carried out as described previously (Jensen *et al.* 1968).

Uterine histomorphology and mRNA levels of interleukin (IL)-1 β and complement C3 in uterus

Frozen sections (5 μ m) were stained with hematoxylin and eosin and 20 sections of each sample (four uteri in each genotype group) were evaluated under a light microscope. The mRNA levels of IL-1 β and complement C3 in the uterus were determined as previously described (Weihua *et al.* 2000).

Results

Effects of estrogen in DERKO mice

The effects of estrogen in mice with both ER α and ER β inactivated (DERKO) were compared with the effects of

Table 2 Effects of estrogen on estrogen-responsive parameters

	WT			DERKO			ERα/ERβ	
	Vehicle	Estrogen	% E over V	Vehicle	Estrogen	% E over V	Dependent	Independent
Trabecular BMD								
Femur (mg/cm ³)	104 ± 10	317 ± 34**	204 ± 33	102 ± 7	120 ± 7	17 ± 7††	92%	8%
Humerus (mg/cm ³)	15 ± 5	389 ± 44**	158 ± 29	141 ± 5	165 ± 15	17 ± 11††	89%	11%
Cortical bone (femur)								
BMC (mg/mm)	0.79 ± 0.02	0.98 ± 0.01**	23.7 ± 1.4	0.84 ± 0.01	1.02 ± 0.02**	21.7 ± 2.2	8%	92%
Area (mm ²)	0.73 ± 0.02	0.86 ± 0.01**	16.5 ± 1.2	0.76 ± 0.01	0.88 ± 0.01**	14.7 ± 2.0	11%	89%
Thymus								
Cellularity	221 ± 25	45 ± 8**	− 79.8 ± 3.4	193 ± 12	159 ± 9*	− 18.1 ± 4.5††	77%	23%
Weight (mg/g)	4.7 ± 0.1	1.4 ± 0.1**	− 71.3 ± 2.0	4.4 ± 0.1	3.3 ± 0.2**	− 24.3 ± 4.4††	66%	34%
Liver								
Weight (mg/g)	42.5 ± 2.3	47.7 ± 1.8*	12.2 ± 3.1	38.6 ± 0.8	45.2 ± 1.3**	17.1 ± 3.5	0%	100%
Fat								
Weight (mg/g)	15.5 ± 1.2	10.9 ± 0.5**	− 29.8 ± 3.3	23.5 ± 0.6	23.0 ± 1.2	− 2.0 ± 5.2††	93%	7%
Uterus								
Weight (mg/g)	0.5 ± 0.1	7.3 ± 0.7**	1319 ± 142	0.2 ± 0.01	1.1 ± 0.1**	558 ± 51††	58% (85%)	42% (15%)

Two left-hand sections: Effects of estrogen (E) on bone, thymus, liver, fat and uterus in ovariectomised (ovx), wild-type (WT) and DERKO mice given both as actual data (vehicle and estrogen) and % increase over vehicle-treated mice (% E over V). Three-month-old ovx mice were treated for 3 weeks with 17β-estradiol or olive oil as control (=vehicle) (n=7–14). Weight values are given as mg/g body weight. Values are given as means ± SEM. *P<0.05, **P<0.01 compared with vehicle-treated mice; ††P<0.01 % effect of estrogen in DERKO compared with the effect of estrogen in WT (Student's t-test). Right-hand section: Calculations of ERα/ERβ-dependent and -independent effects of estrogen. The % effects of estrogen in WT and DERKO mice, as described in the left-hand part of the Table, were used for calculation of the relative proportion of ERα/ERβ-dependent and -independent effects of estrogen. The dependent effects are calculated as effect in (WT – DERKO)/WT × 100. The remaining effect (100-dependent effect) is considered ERα/ERβ independent. Since the vehicle-treated DERKO uteri were much smaller than the vehicle-treated WT uteri (see Figs 4B and 5A), the absolute proportion of the ERα/ERβ-dependent and -independent effects of estrogen on uterine weight is also given in brackets).

Figure 1 Effects of estrogen in DERKO mice. (A) Representative dual X-ray absorptiometry (DXA) scans of the diaphyseal region of the femur. H=high bone mineral density (BMD), L=low BMD, V=vehicle, E=17 β -estradiol. (B) Cortical bone mineral content (BMC), (C) cortical cross-sectional area and (D) cortical thickness as measured by a mid-diaphyseal peripheral quantitative computerized tomography (pQCT) scan of the femur. Three-month-old ovariectomized (ovx) mice were treated for 3 weeks with 17 β -estradiol or the vehicle ($n=7-14$). Values are given as means $\pm$ S.E.M. ** $P < 0.01$ compared with vehicle treatment (Student's t -test).

estrogen in WT mice. Estrogen treatment of ovx WT mice resulted in an increase in the trabecular BMD and cortical bone dimensions as well as in liver and uterine weight while the thymus weight and cellularity and gonadal fat mass were reduced (Table 2). Interestingly, DERKO mice exhibited an unchanged estrogenic response with regard to the increase in cortical bone dimensions and liver weight, as compared with estrogen-treated WT mice (Table 2). The effect of estrogen on femoral cortical bone parameters was similar in WT and DERKO mice (Fig. 1). This cortical effect was due to an increased cortical thickness (Fig. 1D), resulting in an increased cortical cross-sectional area (Fig. 1C) and increased cortical BMC (Fig. 1A and B). DNA microarray analysis was performed to further characterize the molecular nature of the cortical bone effect in DERKO mice. Four probe sets, representing three genes and one EST clone (GenBank no. AI850558, 90% homology with rat $\alpha 2$ -macroglobulin), were found to be increased by estrogen in ovx DERKO mice (Table 3). They were also regulated in a similar manner by estrogen in ovx WT mice. To further investigate the nature of the effect of estrogen on bone and liver in DERKO mice, immunohistochemistry and sucrose gradient analysis were per-

formed. Western immunoblots demonstrated ER α immunoreactivity in liver and bone samples from ER $\alpha^{+/+}$ (WT and BERKO) but not ER $\alpha^{-/-}$ (ERKO and DERKO) mice (Fig. 2A and B). Sucrose gradient analysis of cytosolic extracts from liver samples from WT mice demonstrated a peak with a sedimentation value similar to that of ER α , while no specific estrogen binding was seen in samples from DERKO mice (Fig. 2D). Neither bone samples from WT mice nor bone samples from DERKO mice demonstrated any specific estrogen binding as determined by sucrose gradient analysis (data not shown).

Estrogen exerts ER α /ER β -dependent effects

In contrast to the effects on cortical bone and liver, the estrogenic response to several other parameters, including the trabecular BMD, thymic atrophy, gonadal fat mass and uterine weight were absent or largely reduced in DERKO mice (Table 2). Thus, these parameters were clearly ER α /ER β dependent. The ER specificity was further investigated by comparing the magnitude of the effect in WT, ERKO, BERKO and DERKO mice simultaneously in the same experiment.

Table 3 DNA microarray analysis of the effect of estrogen in ovx DERKO mice. List of mRNAs that are increased >2.5-fold by estrogen in DERKO mice

GenBank accession	Gene description	Mean fold increase	
		DERKO	WT
L20232	Bone sialoprotein (BSP)	3.6	2.0
X72795	Matrix metalloproteinase 9 (MMP-9)	2.8	1.8
X66976	α 1 chain of collagen VIII	3.9	5.1
AI850558	EST clone (90% homology with rat α -macroglobulin)	6.0	6.5

RNA from humerus from six different mice was prepared from each animal group (vehicle-treated WT, estrogen-treated WT, vehicle-treated DERKO and estrogen-treated DERKO). For microarray analysis, the six RNA samples were pooled in two groups of three each, resulting in two pools per animal group. Comparisons were made between the two vehicle-treated and the two estrogen-treated Gene Chips, generating a total of four comparisons for each genotype. An average fold change of the four comparisons was calculated. Four probe sets were identified as increased by estrogen in DERKO, using the strict criteria described in Materials and Methods. As indicated, all these four probe sets were also regulated in a similar manner by estrogen in WT mice. The effects of estrogen on mRNA levels for all four genes were confirmed by real-time PCR analysis (data not shown).

Figure 2 Estrogen receptor (ER) α immunoreactivity and estrogen binding. Western immunoblotting for ER α in (A) bone and (B) liver from WT, ERKO, BERKO and DERKO mice. Sucrose gradient analysis on (C) uterine and (D) liver extracts of WT (◆) and DERKO (○) mice. A magnification of the marked area is shown in the insert in (D). Cytosols were layered onto 10–30% sucrose gradients and centrifuged to equilibrium, and fractions were collected and counted. The arrows indicate the location of a sedimentation value similar to that of ER α as determined by simultaneous analysis of cytosols from MCF-7 cells (data not shown).

Figure 3 Estrogen exerts $ER\alpha/ER\beta$ -dependent effects. (A) Increase in femur trabecular BMD, (B) reduction in thymus cellularity, (C) reduction in gonadal fat and (D) increase in uterine wet weight (similar results were seen for uterine dry weight, data not shown). Three-month-old ovx mice were treated for 3 weeks with 17 β -estradiol or the vehicle ($n=7-9$). Values are given as means $\pm$ S.E.M. and expressed as % of the effect in WT mice. * $P<0.05$, ** $P<0.01$ compared with WT mice (Student's t -test).

Regulation of trabecular BMD is mediated via $ER\alpha$

In WT and BERKO mice, estrogen augmented trabecular BMD while no significant effect of estrogen was seen in ERKO or DERKO mice (Figs 3A and 4A). Histomorphometric analysis of bone volume/total volume in the proximal metaphyseal area of tibia showed similar results as were presented for the trabecular volumetric BMD (data not shown). This finding clearly demonstrates that the effect was mainly $ER\alpha$ dependent (Figs 3A and 4A).

ER specificity for the regulation of thymic atrophy

We have demonstrated here that ERKO and DERKO mice display a strongly reduced estrogen-induced thymic atrophy (reduction in thymus cellularity and thymus weight) while no decrease, but rather an increase, in estrogen-induced thymic involution was seen in BERKO, as compared with WT, demonstrating that $ER\alpha$ is the main receptor responsible for thymic atrophy (Fig. 3B and data not shown).

ER specificity for the regulation of fat mass

In the present experiment, the gonadal fat mass was reduced by estrogen in WT and BERKO but not in

ERKO or DERKO, demonstrating that $ER\alpha$ is responsible for this effect (Fig. 3C). Similar to that seen for thymic involution, an increase in estrogenic response was found in BERKO mice (Fig. 3C).

ER specificity for the regulation of uterine weight

The estrogenic response was reduced for both the uterine wet weight and the uterine dry weight in ERKO and DERKO but not in BERKO mice, confirming the importance of $ER\alpha$ for the uterine response (Figs 3D and 4B). Histomorphology of uteri of ovx mice revealed, as expected, atrophied tissue in all of the genotypes (Fig. 4C). Upon estradiol treatment, though ERKO and DERKO uteri showed much less response than those of WT and BERKO, all four genotypes showed uterotrophic response in terms of water imbibition, elongated nucleus of luminal epithelial cells and decreased density of stromal cells (Fig. 4C). When considering the relative increase in uterine wet weight it would appear as if 42% of the effect of estrogen was preserved in DERKO mice (Table 2, Figs 3D and 4B). However, it should be emphasized that the vehicle-treated DERKO mice had much smaller uteri than the vehicle-treated WT and BERKO mice and somewhat smaller uteri than ERKO mice (WT

Figure 4 ER α is of importance for the regulation of trabecular bone and uterus. (A) Effect on trabecular bone after treatment of 3-month-old ovx mice with 17 β -estradiol (E) or vehicle (V) for 3 weeks. The arrow indicates a central area of the proximal tibial metaphysis consisting of trabecular bone. (B) Macroscopic photographs of uteri after treatment of 3-month-old ovx mice with 17 β -estradiol or vehicle for 3 weeks. Scale bars = 5 mm. (C) Uterine histomorphology. Mitotic figures are indicated by arrows. * = Water imbibition underneath epithelium; + = luminal fluid. Scale bars = 50 μ m.

Figure 5 Effects of estrogen on uterine weight and estrogen-responsive genes in the uterus. (A) Effect on uterine wet weight after treatment of 3-month-old ovx mice with 17 β -estradiol (E) or the vehicle (V). Values are given as means $\pm$ S.E.M. (B) Effect of estrogen on IL-1 β and complement C3 expression in uteri from WT and DERKO mice as studied by RT-PCR. Three-month-old ovx mice were treated for 3 weeks with 17 β -estradiol or vehicle. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard.

11.6 $\pm$ 1.3 mg; ERKO 8.0 $\pm$ 1.3 mg; BERKO 10.8 $\pm$ 0.9 mg; DERKO 3.9 $\pm$ 0.3 mg; Figs 4B and 5A). The absolute estrogen-induced increase in uterine weight was much larger in WT and BERKO mice than in ERKO and DERKO mice (Figs 4B and 5A). Thus, most of the effect of estrogen on the uterus is ER α dependent (85%, Table 2). Sucrose gradient analysis of uterine extracts from WT mice demonstrated a large peak with a sedimentation value similar to that of ER α while a very small peak (approximately 4% of the WT peak) was found with uterine extracts from DERKO mice (Fig. 2C). The effect of estrogen on IL-1 β and complement C3 expression, two genes well known to be regulated by estrogen in the uterus, was determined in the DERKO uterus. Both IL-1 β and C3 were induced by estrogen in WT mice, while neither of these two genes was significantly induced by estrogen in DERKO mice (Fig. 5B).

Discussion

Effects of estrogen in DERKO mice

Our results have demonstrated that DERKO mice exhibit an unchanged estrogenic response with regard to cortical

bone dimensions and liver weight, indicating that estrogen exerts some effects independent of ER α and ER β . However, a recent report, which was published during the preparation of the present manuscript, suggests that the ERKO mice used in the present study are not completely ER α inactivated, supported by the fact that they express one or two N-terminally modified ER α transcripts associated with minor remaining ER activity with regard to uterine weight and endothelial nitric oxide production (Pendaries *et al.* 2002). The remaining ER α activity is suggested to be mediated via remaining activation function-2 (AF-2) activity while there is no AF-1 activity left (Pendaries *et al.* 2002). Thus, the ERKO and DERKO mice used in our study might have a functional ER α AF-2, which may result in a minor ER α activity. Thus, the effect of estrogen on cortical bone in DERKO mice might either be due to remaining ER α AF-2 activity or represent an ER α /ER β -independent effect.

The molecular nature of the preserved effect of estrogen on bone in DERKO mice was investigated by global gene expression analysis. DNA microarray analysis identified three genes and an EST clone, which were increased by estrogen in DERKO mice. The estrogen-induced genes in ovx DERKO mice include BSP, MMP-9 and the α 1 chain of collagen VIII. BSP is a protein expressed by osteoblasts and associated with mineralization of bones (Ganss *et al.* 1999). MMP-9 has been described as being expressed by osteoblasts, osteocytes, osteoclasts and macrophages, and has been suggested to be involved in endochondral ossification (McClelland *et al.* 1998, Vu *et al.* 1998). Interestingly, it has previously been demonstrated that bone formation induced by intermittent parathyroid hormone administration is associated with a stimulation of MMP-9 expression in osteoblasts (McClelland *et al.* 1998). Thus, BSP and MMP-9 might be important candidate genes involved in the stimulatory effects of estrogen on cortical bone in mice. The physiological role of collagen VIII in bone is unclear but it has been reported to stimulate the production of MMP-9 (Hou *et al.* 2000). ER α immunoreactivity was found in liver and bone samples from ER α ^{+/+} but not ER α ^{-/-} mice and it has been previously demonstrated that no ER β immunoreactivity is detected in ER β ^{-/-} mice (Krege *et al.* 1998). Furthermore, sucrose gradient analysis on cytosolic extracts from liver detected ER α in WT but not in DERKO mice. These data may indicate that the effect of estrogen on cortical bone and liver in DERKO mice might be ER α /ER β independent but, as described above, one cannot exclude a remaining ER α activity. Alternatively, estrogen has been suggested to exert non-genomic actions via cell membrane receptors in a variety of cell types, including osteoblasts (Nemere & Farach-Carson 1998, Le Mellay *et al.* 1999, Kousteni *et al.* 2001) and the effect in DERKO mice might

also be due to some low affinity binding to other known nuclear receptors (Kousteni *et al.* 2001). However, one cannot exclude the possibility that the effects on bone and liver are indirect, mediated via other mechanisms, which, for instance, might include an effect on the hypothalamic/pituitary axis. The exact mechanism behind the effects of estrogen on cortical bone and liver in DERKO mice remains to be elucidated.

Trabecular BMD effect is mediated via ER α

Estrogen increased the trabecular BMD in ovx WT and BERKO, but not in ERKO or DERKO mice. This finding clearly demonstrates that the effect was ER α dependent. Interestingly, as described above, the other major bone compartment, the cortical bone, did respond to estrogen in DERKO compared with WT mice. Separate mechanisms of action for estrogen in the regulation of the trabecular bone versus the cortical bone are supported by two knockout studies demonstrating that the trabecular bone responded differently to estrogen deficiency compared with cortical bone in plasminogen activator inhibitor-1- and IL-6-deficient mice (Poli *et al.* 1994, Daci *et al.* 2000).

Thymic atrophy and fat mass effects are mediated via ER α while ER β inhibits these effects

The present study confirmed previous studies demonstrating that ER α is the receptor responsible for thymic atrophy and regulation of fat mass (Staples *et al.* 1999, Heine *et al.* 2000, Ohlsson *et al.* 2000). Interestingly, an increased estrogenic response for these two parameters was found in BERKO mice, indicating that ER β might act as a repressor of these ER α -mediated effects.

Uterine weight effect is mainly mediated via ER α

The estrogenic uterine responses in mature ovx WT, ERKO, BERKO and DERKO mice have not previously been compared. Responses were reduced for both the uterine wet weight and the uterine dry weight in ERKO and DERKO but not in BERKO mice, confirming previous studies regarding the importance of ER α for the uterine response (Lubahn *et al.* 1993, Couse *et al.* 1999). However, there was a small but significant remaining uterotrophic effect of estrogen in ERKO and DERKO mice. A similar remaining effect of estrogen in ERKO mice has recently been reported by Pendaries *et al.* (2002) and shown to be dependent on the AF-2 activity of the truncated ER α present in the uterus of our ERKO model. Sucrose gradient analysis of uterine extracts from WT mice demonstrated a large ER α -like peak while a very small peak was found with uterine extracts from DERKO

mice. Thus, it cannot be excluded that the receptor responsible for this small remaining estrogen-binding capacity is involved in the minor uterotrophic effect of estrogen in DERKO mice. Both IL-1 β and C3 were induced by estrogen in WT mice, while neither of these two genes was significantly induced by estrogen in DERKO mice. These measurements indicate that the effect of estrogen in DERKO uterus may, at least partly, differ from that seen in WT mice. Why, then, do vehicle-treated ovx DERKO mice have smaller uteri than vehicle-treated ovx WT mice? This might be explained by an ER α /ER β -dependent stimulation by exogenous estrogens, including diet-derived phytoestrogens or by ER α /ER β being of some importance for the early development of the uterus. We have previously shown that estrogen treatment of young, sexually immature, female mice results in a more pronounced uterine response in BERKO mice compared with WT mice (Weihua *et al.* 2000). In contrast, the uterine response to estrogen was, in the present study, not increased in BERKO mice. However, there are two important differences between our present and our previous study: (1) in the present study the mice were sexually mature while they were immature in the previous study and (2) the mice in the present but not in the previous study were ovariectomized before treatment with estrogen.

ER β represses some ER α -mediated effects

Although ER α appears to be of major importance in the ER α /ER β -dependent responses studied in this investigation, ER β plays an important role in some other physiological contexts. For instance, ER β is indispensable for normal ovarian morphology and function as inferred from ER β knockout studies in mice (Krege *et al.* 1998). Very recently we have also demonstrated, using the same animal model, that ER β is necessary for normal morphology in several regions of the central nervous system (Wang *et al.* 2001). Studies on these animals also indicate that ER β has an antiproliferative effect in the immature uterus and in the prostate, at least partially by balancing the proliferative activity of ER α ('yin-yang effect') (Weihua *et al.* 2000, 2001). It has also been suggested that ER β may repress the expression of ER α (Windahl *et al.* 2001). Thus, the increased effect of estrogen on thymus involution and fat reduction in BERKO mice, observed in the current study, might be explained by an unopposed ER α activity.

In conclusion, estrogen increased the cortical bone dimensions in both WT and DERKO mice. DNA microarray analysis identified four genes that were regulated by estrogen in DERKO mice. The effect of estrogen on cortical bone in DERKO mice might be due either to remaining ER α activity or might represent an ER α /ER β -independent effect. Other effects such as increased trabecular BMD, thymic atrophy, fat reduction and increased uterine weight were mainly ER α mediated.

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