THE RELATIVE IMPORTANCE OF SEX STEROID RECEPTORS FOR BONE METABOLISM

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2005

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AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Sahlgrenska akademin vid Göteborgs universitet kommer att offentligen försvaras i hörsal Arvid Carlsson, Academicum, Medicinaregatan 3, Sahlgrenska universitetssjukhuset, Sahlgrenska

fredagen den 20 januari 2006 kl. 9.00

av
Sofia Movérare Skrtic

Fakultetsopponent: Docent Kenneth B. Jonsson
Uppsala Universitet

Avhandlingen baseras på följande arbeten:

I. Lindberg MK, Movérare S, Skrtic S, Alatalo S, Halleen J, Mohan S, Gustafsson J-Å, Ohlsson C
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ABSTRACT

Estrogen deficiency increases the risk of a wide variety of pathological conditions including osteoporosis. Morbidity from osteoporosis is substantial in both men and women. Hormone replacement therapy (HRT, including estrogens) is associated with side-effects such as increased risk of breast cancer, stroke and deep venous thrombosis, and agents that can maintain the benefit of estrogen but avoid the risks are therefore needed. Testosterone is the precursor molecule of estradiol and its effects are mediated either via a direct activation of the androgen receptor (AR) or indirectly via aromatisation into estradiol and activation of estrogen receptor α (ERα) or ERβ. The aim of this thesis was to compare the relative importance of and the interactions among AR, ERα, and ERβ for bone metabolism in male and female mice.

Male mice Orchidectomy resulted in a pronounced decrease in trabecular bone mineral density (BMD), which was completely prevented by estrogen treatment in wild type (WT) and ERβ$^{-/-}$ but not in ERα$^{-/-}$ or ERα$^{-/-}$β$^{-/-}$ mice. This finding demonstrates that ERα is important while ERβ is of no importance for the regulation of the trabecular BMD in male mice. Furthermore, in an additional study we showed that not only ERα activation but also a specific AR activation preserves the amount of trabecular bone in orchidectomized mice. Micro-computer tomography analyses demonstrated that ERα activation resulted in both preserved thickness and number of trabeculae, whereas AR activation only preserved the number but not the thickness of trabeculae. ERα activation increased serum levels of insulin-like growth factor I, which were positively correlated with all the cortical and trabecular bone parameters specifically preserved by ERα activation, suggesting that insulin-like growth factor I might mediate these skeletal effects of ERα activation. Thus, the *in vivo* bone-sparing effect of ERα activation is distinct from the bone-sparing effect of AR activation in adult male mice.

Female mice By demonstrating that combined ER and AR activation resulted in a more pronounced effect on the trabecular bone than the single treatments in ovariectomized mice, we extended our previous findings that ERα, ERβ as well as AR are of importance for the trabecular bone in female mice. Because the ER and the AR pathways are distinct from each other, a combined treatment of selective ER modulators and selective AR modulators might be beneficial in the treatment of osteoporosis. By investigating global estrogen-regulated gene transcription in ovariectomized mice, we found that ERβ inhibits ERα-mediated gene transcription in the presence of ERα, whereas in the absence of ERα ERβ can partially replace ERα. These findings indicate that one important physiological role of ERβ is to modulate ERα-mediated gene transcription, supporting a "Yin Yang" relationship between ERα and ERβ in mice.

In conclusion, ERα and AR-activation increases the amount of trabecular bone by distinct mechanisms while ERβ is of no importance for the trabecular bone in male mice. In female mice, AR and ER-activation results in an additive effect on trabecular bone mass and ERβ modulates ERα-mediated gene transcription in bone.

Keywords: bone, estrogen, androgen, transgenic, microarray

ISBN 91-628-6688-5
Göteborg 2005

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Printed by Intellecta Docusys
Göteborg, Sweden, 2005

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List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

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Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
aBMD	areal bone mineral density
AF-1/2	activation function 1/2
ALP	alkaline phosphatase
ANGELS	activators of nongenotropic estrogen–like signaling
AP-1	activating protein 1
AR	androgen receptor
ARE	androgen responsive element
BERKO	estrogen receptor β knockout ($ER\beta^{-/-}$)
BMC	bone mineral content
BMD	bone mineral density
BMU	basic multicellular unit
BV/TV	trabecular bone volume over total bone volume
C/EBPβ	CCAAT/enhancer binding protein β
CBP	CREB-binding protein
CCD	charged-coupled detector
cDNA	complementary DNA
CRE	cyclic AMP-response element
CREB	cAMP-response element binding protein
CYP19	cytochrome p450 aromatase
DBD	DNA binding domain
DERKO	double estrogen receptor knockout ($ER\alpha^{-/-}ER\beta^{-/-}$)
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
DRIP	vitamin D receptor-interacting protein
DXA	dual x-ray absorptiometry
EGF	epidermal growth factor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERE	estrogen responsive element
ERK	extracellular-signal regulated kinase
ERKO	estrogen receptor α knockout ($ER\alpha^{-/-}$)
EST	expressed sequence tag

Estren	estren-3α,17β-diol
GPR30	G protein-coupled receptor 30
IGF-I	insulin-like growth factor-I
IL	interleukin
JNK	c-Jun N-terminal kinase
KGF	keratinocyte growth factor
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
μCT	micro-computer tomography
mRNA	messenger RNA
NCoR	nuclear receptor corepressor
NTD	N-terminal domain
Orx	orchidectomy
Ovx	ovariectomy
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
pQCT	peripheral quantitative computerised tomography
PTH	parathyroid hormone
SARM	selective androgen receptor modulator
SERM	selective estrogen receptor modulator
SMRT	silencing mediator of retinoid and thyroid receptors
SNF	sucrose non-fermenting
SP-1	specificity protein 1
SRC	steroid receptor coactivators
SWI	mating type switching
Tb.N.	trabecular number
Tb.Sp.	trabecular separation
Tb.Th.	trabecular thickness
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
TRAP	thyroid-hormone receptor associated protein
vBMD	volumetric bone mineral density
WT	wild type

Introduction

General introduction

Loss of bone mass along with microarchitectural deterioration leads to enhanced bone fragility and increased fracture risk — the bone disease known as osteoporosis (1). Osteoporosis affects both women and men, with a lifetime fracture risk of 40% in women and 20% in men, and the risk is likely to increase with longer life expectancy (2). After sex and age, the major risk factor for osteoporosis is estrogen deficiency. Estrogen replacement therapy is associated with side-effects such as breast cancer, stroke and deep venous thrombosis. Therapeutic agents that can maintain the benefit of estrogen but avoid the risks are therefore needed. The development of such agents requires increased knowledge about the mechanism behind sex steroid action.

Bone

The function and structure of the skeleton

Bone is specialized connective tissue which, together with cartilage, makes up the skeletal system. This tissue serves three functions: (i) mechanical, as support and site of muscle attachment for locomotion, (ii) protective, for vital organs and bone marrow, and (iii) metabolic, as a reserve for ions, especially calcium and phosphate (3). The skeleton contains of two different types of bone, cortical and trabecular bone. They consist of the same cells and matrix elements, but there are structural and functional differences. Eighty to ninety percent of the volume of cortical bone is calcified, whereas only 15-20% of the trabecular bone is calcified. This results in the major difference in function: cortical bone has mainly a mechanical and protective function whereas trabecular bone plays a more metabolic role, since more of its surface interfaces with soft tissue (3). The bone cells are: the osteoclasts, the bone resorbing cells; the osteoblasts, the bone forming cells responsible for production of the matrix constituents, and the osteocytes, originally osteoblasts entrapped in the matrix they produced (4-7). The extracellular matrix consists of collagen fibres, mainly type I, and noncollageneous proteins such as osteocalcin and alkaline phosphatase (ALP) (3).

Structure of the long bones

The long bones can be divided into three anatomical parts, the diaphysis, the metaphysis, and the epiphysis (3) (fig. 1). The diaphysis consists of cortical bone surrounding the bone marrow cavity. The outer part of the cortical bone is surrounded by the periosteum and the inner part by the endosteum. The epiphysis and the metaphysis consist of trabecular bone surrounded by a thin layer of cortical bone. They are separated by a layer of cartilage called the epiphyseal cartilage or the growth plate.

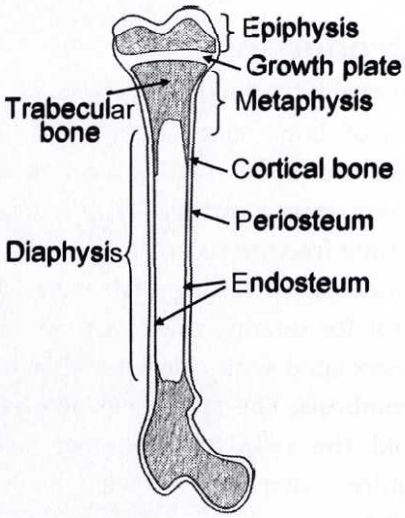

Fig. 1. Schematic view of a longitudinal section through a long bone.

Bone formation

Intramembranous ossification involves the replacement of sheet-like connective tissue membranes with bony tissue (3, 8). Bones formed in this manner include certain flat bones of the skull and some facial bones. The bones are first formed as connective tissue membranes. Osteoblasts migrate to the membranes and deposit bony matrix around them. Endochondral ossification involves the replacement of cartilage with bony tissue. Most of the bones of the skeleton, for instance the long bones, are formed in this manner. In this process, bones are first formed as cartilage models. The cartilage models become infiltrated with blood vessels and osteoblasts and a periosteum emerge. The osteoblasts form a collar of compact bone around the diaphysis and at the same time the cartilage in the centre of the diaphysis begins to disintegrate. Osteoblasts penetrate the disintegrating cartilage and replace it with spongy bone, which forms a primary ossification centre. Ossification continues from this centre toward the ends of the bones. After spongy bone is formed in the diaphysis, osteoclasts break down the newly formed bone to open up the medullary cavity. The cartilage in the epiphysis continues to grow so the developing bone increases in length. Later, usually after birth, secondary ossification centres form in the epiphysis. When secondary ossification is complete, the cartilage is totally replaced by bone,

except at the articular cartilage over the surface of the epiphysis and at the growth plate.

Longitudinal growth

Bones grow in length at the epiphyseal growth plate by a process that is similar to endochondral ossification. The cartilage in the region of the epiphyseal growth plate next to the epiphysis continues to grow by mitosis. The chondrocytes, in the region next to the diaphysis, begin to proliferate, and subsequently the chondrocytes differentiate and become hypertrophic. Following the mineralization process the chondrocytes undergo apoptosis (9, 10). Osteoblasts move in and ossify the matrix to form bone. This process continues throughout childhood. At puberty, a growth spurt occurs, followed by a rapid decrease in growth velocity, attributable to growth plate maturation in long bones and spine, and leading to growth plate fusion and cessation of longitudinal growth (11, 12). Longitudinal bone growth is under the influence of a multitude of genetic and hormonal factors, growth factors, environment, and nutrition (13-16).

Bone remodeling

The bone mass increases until the age of 20-25, when it reaches its maximum value, peak bone mass (17-21). Once bone formation has ceased, peak bone mass is maintained by a process known as bone remodeling (22). This process is thought to enable the bone to respond and adapt to mechanical stress and also to repair damage to maintain strength. Old bone is removed (resorption) and new bone is created (formation). To maintain constant bone mass, bone formation and resorption have to be in perfect equilibrium. This is achieved by the coupling mechanism between the osteoblasts and the osteoclasts. However, after a certain age bone mass begins to decrease which can lead to osteoporosis and an increased risk of fracture. The bone remodeling process is cyclical with different phases taking place in basic multicellular units (BMUs) (23) (fig. 2). In the activation phase, preosteoclasts are stimulated and differentiate into active osteoclasts. During the subsequent resorption phase, the osteoclasts digest mineral matrix. In the intermediate phase, known as the reversal phase, resorption ends and osteoblasts migrate to the resorption site. During the formation phase, the osteoblasts produce matrix, which is subsequently mineralised. The whole remodeling cycle is under the control of cytokines, growth factors and hormones such as interleukin (IL)-1, TNFα, IL-6, TGFβ, PTH, and vitamin D_3 (22). In humans, the completion of one

cycle takes 6-9 months and each year 3-4 million cycles are initiated, leading to complete regeneration of the skeleton approximately every ten years.

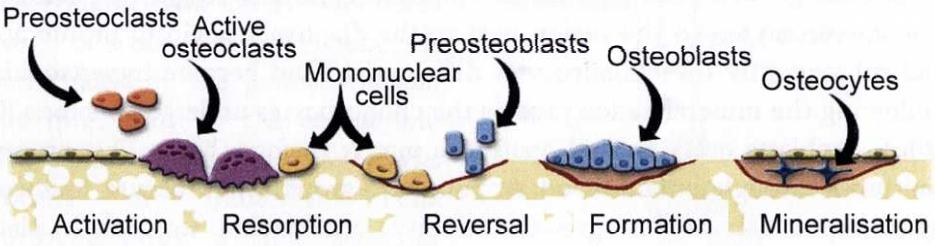

Fig. 2. The bone remodeling cycle. The cycle starts with activation of the preosteoclasts which, under the influence of cytokines and growth factors, differentiate and mature into active osteoclasts. In the resorption phase, the osteoclasts digest mineral matrix. This phase is followed by a reversal phase where the osteoclasts undergo apoptosis, and preosteoblasts become mature osteoblasts. Osteoblasts synthesize new bone matrix in the formation phase, and when the cavity is filled, osteoblast undergo apoptosis or turn into osteocytes or lining cells. The new bone becomes more densely mineralised for up to three years.

Sexual dimorphism of the skeleton

Adult men have greater bone mass than women, owing to greater bone volume, not greater volumetric bone mineral density (vBMD). At puberty, men develop bigger bone size than women, owing to increased periosteal apposition. In women, estrogens have an inhibitory effect on the periosteal bone formation. Furthermore, the epiphyseal closure is stimulated earlier by estrogens in women then in men. After puberty, the sexual dimorphism regarding bone size and bone mass is further enhanced, owing to increased periosteal apposition in males (24, 25).

Sex steroids and their receptors

Synthesis and metabolism of sex steroids

In premenopausal women, more than 95% of the estradiol in the circulatory system is derived from ovarian secretion. In young adult men, more than 95% of serum testosterone is derived from testicular secretion (26). However, in postmenopausal women and elderly men, the gonadal secretion of sex steroids decreases and the main source of sex steroids is extragonadal conversion of C19 androgen precursors in the target tissue. The adrenal cortex and the gonads secrete large amounts of C19 androgens, mainly dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and Δ^4-androstenedione (27, 28) (fig. 3). Although only weakly androgenic themselves, they are an important source of substrate for the extragonadal synthesis of potent sex steroids. In the periphery, C19 androgens are converted into testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). In many target tissues, 5α-dihydrotestosterone (DHT), irreversibly formed from testosterone through the action of the enzyme 5α-reductase, is the main source of androgenic activity. Testosterone can also be aromatised into estradiol by the enzyme cytochrome p450 aromatase (CYP19).

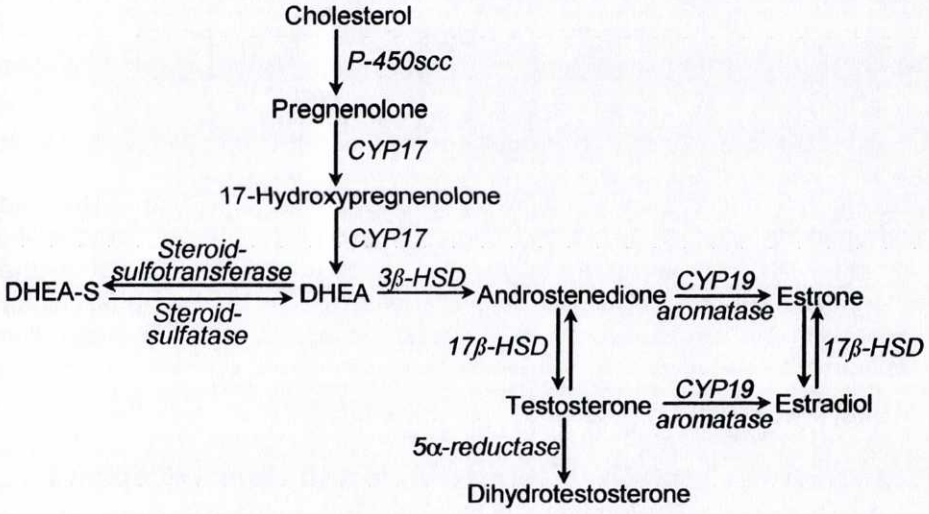

Fig. 3. Sex steroid synthesis. Testosterone can be converted into dihydrotestosterone by 5α-reductase or it can be aromatised into estradiol by CYP19. 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; CYP19: cytochrome p450 (aromatase); DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone sulfate.

Structure of the sex steroid receptors

Both the two estrogen receptors ERα and ERβ as well as the androgen receptor AR belong to a superfamily of nuclear hormone receptors including those for other steroid hormones, thyroid hormone, vitamin D, and retinoic acid. The ERα was cloned in 1986 (29), AR in 1988 (30, 31), and ERβ in 1996 (32). Nuclear receptors are ligand-activated transcription factors that possess quite divergent N-terminal domains (NTDs) (A/B), highly conserved DNA-binding domains (DBDs) (C), a hinge region (D), and moderately conserved ligand-binding domains (LBDs) (E) (33) (fig. 4). The NTD encodes the ligand-independent activation function 1, AF-1 (34). The LBD region is responsible for the dimerisation of the protein, and this region also harbours AF-2, which is a complex region whose structure and function are governed by the binding of ligands (34). ERβ appears to have no significant AF-1 activity, and thus depends entirely on the ligand-dependent AF-2 (35-37).

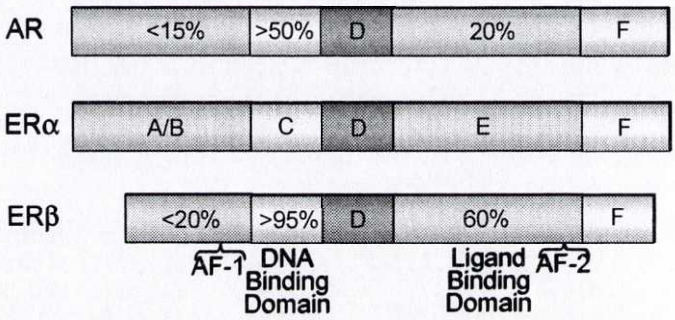

Fig. 4. Schematic representation of estrogen receptor (ER)α, ERβ, and androgen receptor (AR). Nuclear receptors consist of five characteristic domains, A-F. Numbers represent percent of homology to ERα in the different domains. The DNA binding domain (C) is the most highly conserved domain between the nuclear receptors. AF-1/2: activation function 1/2.

Mechanisms of action of sex steroids and their receptors

Sex steroids are known to influence the expression of a wide range of genes by different mechanisms (38) (fig. 5). The most common, classic means of action is via a ligand-dependent activation of the specific receptor, but it is now well known that other mechanisms are also possible, such as ligand-independent or DNA-binding-independent activation. Gene transcription

can also be activated by steroids via activation of a non-genomic signaling cascade.

ER/AR and classic genomic activity

Classically, these receptor proteins function as transcriptional factors in the nucleus when they are bound to their respective ligands (39). Hormone binding to the receptor activates the protein through phosphorylation. The receptor conformation is altered and chaperone proteins such as heat-shock protein 90 dissociate. The hormone-bound receptor then dimerises with another receptor, and the dimer binds to specific DNA sequences, i.e. estrogen or androgen responsive elements, ERE and ARE, respectively, present in the promoter of the responsive genes. Promoter-bound receptor dimers form a complex with coregulatory proteins that act coordinated to influence the transcription of the responsive genes (40, 41). Current estimates reveal that there are approximately 150 different coregulators that have been suggested to play a role in nuclear receptor actions in cells (42). Most characterized are coregulators of the steroid receptor coactivator (SRC)-1/p160 family; the acetyltransferases cyclic AMP-response element binding protein (CREB)-binding protein (CBP)/p300; the thyroid hormone receptor associated protein (TRAP)/vitamin D receptor-interacting protein (TRAP/DRIP) complex; the ATP-coupled chromatin remodeling SWI/SNF complex; and the complex of corepressor proteins including silencing mediator of retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NCoR) (reviewed in (42, 43)).

ER/AR and ligand-independent transcriptional activity

The ER/AR function can also be modulated by extracellular signals in the absence of a hormone. This signal transduction is mediated via cross-talk between the receptors and growth factor receptors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and insulin-like growth factor-I (IGF-I) receptors (44-50). Although the mechanism behind this modulation of gene and subsequent protein expression is not completely understood, the majority of evidence indicates that modification of the phosphorylation state of the ER/AR by cellular kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) serves as an important mechanism of ligand-independent activation (51-53).

ER/AR and non-classic transcriptional regulation

ERs and AR have also been shown to modulate gene expression at alternative regulatory DNA sequences such as activating protein (AP)-1, specificity protein 1 (SP-1), variant cyclic AMP-response elements (CREs), Smad3 and upstream stimulatory factor sites, as well as other poorly defined non-ERE/ARE sites (54-58). In these circumstances, ERs/AR do not function as the major transcription factor but rather are tethered to the specific promoter complex by its interaction with other DNA-bound transcription factors such as c-Jun and c-Fos, or with other coactivator proteins. Transcription of several genes important to growth factor signaling transduction pathways is regulated in this way.

Sex steroids and non-genomic activity

Sixty years ago it was reported that steroid hormones might have very rapid action on cells, too rapid to invoke transcriptional mechanisms (59). Binding sites for estrogen has been identified in the membrane of endometrial cells that trigger the induction of cyclic AMP (60). Binding sites for androgens have also been found in plasma membranes (61-63). Later studies have also argued for the presence of sex steroid receptors outside the nucleus that can mediate rapid signals originating from the membrane or in the cytoplasm (64). Recently, GPR30, a G protein-coupled receptor localised to the plasma membrane or the endoplasmic reticulum, has been found to be activated by estrogen (65-67). This nongenomic action or membrane-initiated steroid signaling occurs within minutes of the addition of the hormone. The membrane-initiated steroid signaling results in activation of conventional second messenger signal transduction cascades, including activation of protein kinase A (PKA), protein kinase C (PKC), cellular tyrosine kinases, MAPKs, PI3K and Akt-signaling enzymes and adaptors such as adenyl cyclase and Sch (68-70).

Although it has become increasingly clear that sex steroids and their receptors are involved in a wide diversity of cellular pathways, it is important to bear in mind that the truth is even more complex, as the cellular pathways are often connected downstream of the signaling cascade. Furthermore, different pathways are important in different tissues and cell stages, and their significance regarding bone metabolism has not yet been clarified.

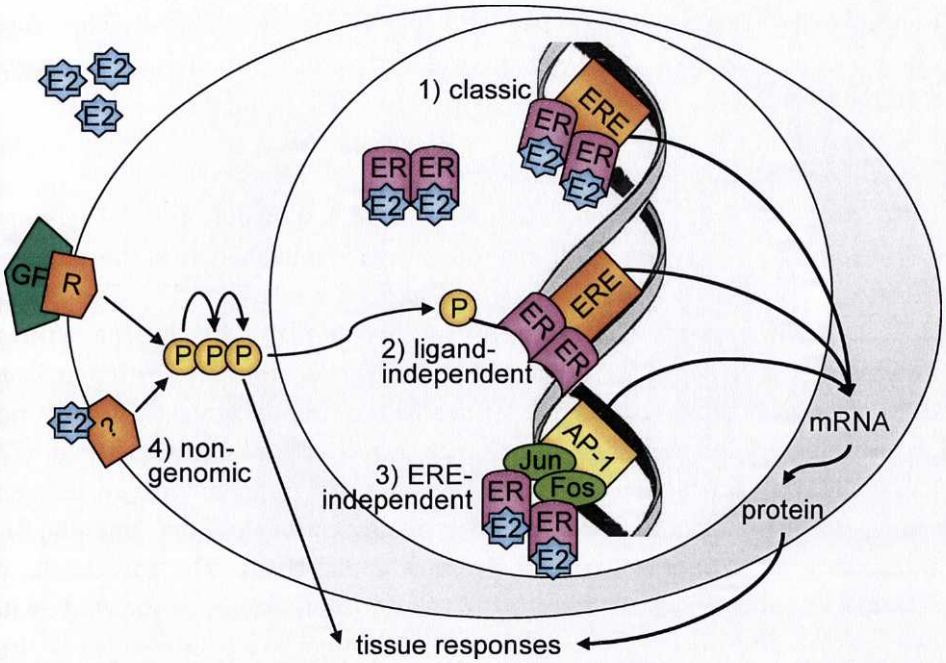

Fig. 5. Mechanisms of sex steroid signaling represented here by estrogen (E2) and estrogen receptor (ER). The effects of E2 are mediated through at least four pathways. *1) classic ligand-dependent*, E2-ER complexes bind to EREs in target promoters. *2) ligand-independent*, for instance growth factors (*GF*) activate intracellular kinase pathways, leading to phosphorylation (P) and activation of ER at ERE-containing promoters in a ligand-independent manner. *3) ERE-independent*, E2-ER complexes alter transcription of genes containing alternative response elements such as AP-1 through association with other DNA-bound transcription factors (Jun/Fos). *4) Non-genomic signaling*, E2 activates a putative membrane-associated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses.

ERβ can function as a repressor of ERα

Previous *in vitro* data has indicated that ERβ can function as a dominant negative regulator of ERα activity. When complexed with estradiol, ERα and ERβ signal in opposite directions from an AP-1 site (71). Furthermore, transient transfection assays have demonstrated that ERβ has the capacity to repress the transcriptional activity of ERα (36, 72). *In vivo* it has been demonstrated that female ERβ inactivated mice have increased amounts of cortical bone and are partly protected against age-related trabecular bone loss

that normally occurs in old female wild type (WT) mice (73, 74). Therefore, one may speculate that ERβ represses the bone-protection effects of estrogen mediated via ERα.

Effects of sex steroids on the skeleton

Until recently, androgens were assumed to be responsible for the pubertal growth spurt (75). However, it has now been established that the pubertal growth spurt of both sexes is driven primarily by estrogen (76). Estrogen is also responsible for the closure of the growth plate. In the few known examples of men who do not have the ability to aromatise estrogen from testosterone or with a disruption in the ERα gene, the growth plate is not properly closed and the longitudinal growth is therefore not interrupted (77-79). Estrogen treatment closes the growth plate in men with a deficient aromatase gene but not in the man with disruption in the ERα gene (80, 81). Estrogen is also important to adult bone metabolism. The low levels of estrogen due to ovarian failure following menopause, associated with osteoporosis and higher rates of fractures are the clearest indications of this. Estrogen has negative effects on bone resorption, although stimulatory effects on osteoblasts and bone formation have also been reported (82-84). More and more evidence has revealed that estrogen is important to both the female and male bone metabolisms. Adult men who cannot produce or respond to estrogen are osteopenic, and treatment with estrogen increases the bone mass of aromatase deficient men (78-80). Although men do note have the rapid phase of bone loss present at menopause in women, they still lose substantial amounts of bone as they age (85-88). Increased bone resorption in elderly men can be prevented by estrogen treatment (89, 90).

The effects of sex steroids on the skeleton have been widely studied in rodents by use of castration, administration of ER antagonists, AR antagonists, aromatase inhibitors, selective estrogen receptor modulators (SERMs), and type II 5α-reductase inhibitors, and definitely also by use of transgenic mice. Gonadectomy reduces serum levels of androgens and estrogens, and this is associated with an increase in trabecular bone turnover, where bone resorption is more pronounced than bone formation, resulting in bone loss (91-95). Regarding the cortical bone, gonadectomy has a sexual dimorphic pattern. In males, orx decreases bone mass whereas in females the cortical bone mass in unaffected or increased after ovx (92, 96). Both aromatisable testosterone, the non-aromatisable DHT as well as estrogen have bone-sparing effects on the trabecular bone (93, 94, 97-100). It is clear

that both estrogen and androgen are important for the skeleton, although their relative significance has not been studied.

Selective Estrogen and Androgen Receptor Modulators (SERMs/SARMs)

In recent years, estrogen has been one of the most frequently prescribed drugs in the world. However, estrogen use has declined sharply in the wake of the early termination of the Women's Health Initiative (WHI) in 2002, once it became clear that the risks exceeded the benefits (101). SERMs are a new category of therapeutic agents available for the prevention and treatment of diseases such as osteoporosis and breast cancer. Unlike estrogens, which are uniformly agonistic, and antiestrogens, which are uniformly antagonistic, SERMs display an unusual tissue-selective pharmacology (102). They are agonistic in some tissues (bone and liver), antagonistic in other tissue (breast), and combined agonistic/antagonistic in the uterus.

There is a great deal of hope that SERMs will mimic the essential benefits of estrogen for the bones at the same time as they will act as antiestrogens in the breast and uterus, thus avoiding the harmful effects of estrogen in these tissues. Most of the unique pharmacology of SERMs can be explained by three interactive mechanisms: (i) differential ER expression in a given target tissue, (ii) differential ER conformation on ligand binding, and (iii) differential expression and binding to the ER of coregulator proteins (103, 104). Although the use of SERMs is widespread, there has not been a similar trend observed for androgen therapies. SARMs behave as partial agonists in androgenic tissues (prostate and seminal vesicle), and as full agonists in anabolic tissues (levator ani muscle). For osteoporosis indications, a SARM with anabolic activity in bone and possibly muscle, but with relatively little activity on sex-accessory tissues, would be desirable. The role of SARMs regarding osteoporosis is under investigation, but they are not yet used in clinical practice.

Estren – activator of non-genotropic estrogen-like signaling (ANGELS)?

Recently, a new synthetic compound, estren-3α,17β-diol (estren), has been presented by the group of Dr. Manolagas (70, 100, 105). Estren has been suggested to be a mechanism-specific ligand of the ERs or the AR rather than being a tissue-specific ligand such as SERMs/SARMs. The term ANGELS has been coined for ER ligands that activate nongenotropic estrogen–like signaling, but that lack the ability to induce the classic transcriptional

activity of the ER. Estren is a four-ring synthetic steroid analogue prototypical compound of ANGELS proposed to activate MAPKs and PI3K. This non-genomic pathway is suggested to be of importance for the trabecular BMD, whereas no effect of the compound has been found on the reproductive organs.

Aims of the thesis

- To investigate the relative roles of sex steroid receptors in the regulation of the bone metabolism in male mice (papers I-II).

- To investigate the effects of combined activation of both the ERs and the AR on the female rat skeleton (paper III).

- To investigate the role of ERβ and its possible interactions with ERα in global estrogen-regulated transcriptional activity (paper IV).

- To further characterise the synthetic compound estren, regarding its tissue specificity and transcriptional activity (paper V).

Methodological considerations

The methods used in this thesis are described in the Materials and Methods sections of the individual papers. A more general discussion of some of the methods is presented below.

Animal models

Both rats and mice are used to study the effects of different treatments on the skeleton. Rats were initially most often used due to their larger size, but as a result of increased knowledge about transgenic mice, they are more commonly used today. Transgenic mice have one gene that is inactivated or overexpressed and the function of the specific gene can thereby be studied.

One difference between the human and rodent skeleton is the closure of the growth plate, which takes place directly after puberty in humans. In rodents, the growth plate is never fully closed (106), but with increasing age, its characteristics stabilize and do not change significantly (107, 108). Furthermore, the coupling between the osteoblasts and osteoclasts which is crucial for the maintenance of the bone mass is independent of longitudinal growth and rodents may therefore be used as a model of adult human bone remodeling.

$ER\alpha^{-/-}$ (ERKO), $ER\beta^{-/-}$ (BERKO) and $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) transgenic mice

The generation of $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) is somewhat complex, since female $ER\alpha^{-/-}$ (ERKO), $ER\beta^{-/-}$ (BERKO) and male $ER\alpha^{-/-}$ (ERKO) mice are infertile. Double heterozygous mice ($ER\alpha^{+/-}ER\beta^{+/-}$) are mated, resulting in WT, $ER\alpha^{-/-}$ (ERKO), $ER\beta^{-/-}$ (BERKO) and $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) offsprings. This breeding results in one $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) pup of the right sex out of 32 offspring. The $ER\alpha^{-/-}$ (ERKO) mice used in this thesis were not completely ERα-inactivated. The mice express N-terminally modified transcripts generating a truncated ERα with remaining AF-2 activity, while AF-1 is absent (109). A second $ER\alpha^{-/-}$ (ERKO) model lacking both AF-1 and AF-2 has been generated by the lab of Chambon (110). However, most skeletal phenotypes are identical for these two transgenic models, except as concerns female gonadal intact mice.

Gonadectomy and hormonal treatment

Surgical castration, as induced by ovariectomy (ovx) in female rodents and orchidectomy (orx) in male rodents, represents the most frequently used

procedure for studying skeletal sex steroid action. Both ovx and orx dramatically reduce serum levels of testosterone and estrogen in rodents. However, since adrenal androgens can be transformed into estrogens after aromatisation, these procedures do not totally eliminate estrogen production. Gonadectomy is associated with a decrease in BMD, which corresponds to the decline in BMD seen in postmenopausal women and castrated men.

The doses of 17β-estradiol and DHT used in this thesis have been carefully chosen to correspond to normal serum levels of the hormones in the circulatory system. For 17β-estradiol, this correspond to a preserved size of the uterus after ovx and for DHT treatment the dose chosen correspond to a preserved size of the seminal vesicles and the ventral prostate after orx. The dose of estren used in paper IV was chosen as being slightly lower than the dose used by Kousteni et al. (105).

Measurements of bone parameters

The methods mentioned below regarding measurements of bone parameters are illustrated in fig. 6.

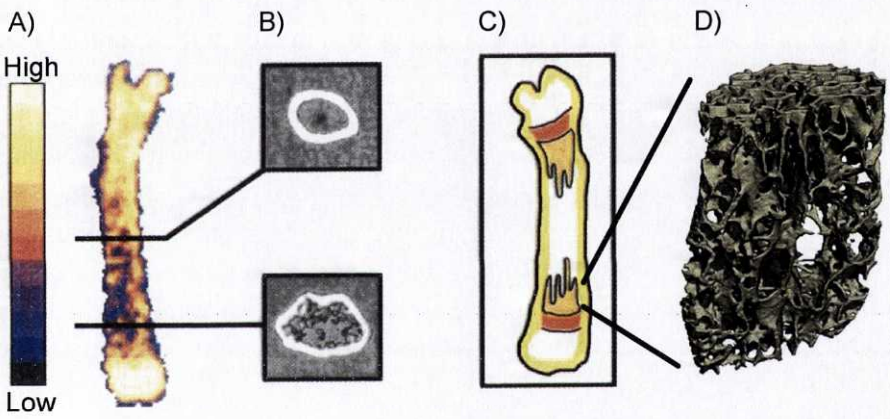

Fig. 6. Techniques for bone measurements. A) dual x-ray absorptiometry (DXA), B) peripheral quantitative computer tomography (pQCT), C) bone histomorphometry, and D) micro-computer tomography (μCT). DXA and bone histomorphometry are 2D techniques while pQCT and μCT are 3D techniques. pQCT can measure both the cortical (upper image), and the trabecular bone (lower image). μCT is the technique with highest resolution, 5 μm, which makes it possible to study separate trabeculae.

Histomorphometry

Histomorphometry is the classic method used to study bone parameters. The bone of interest is embedded in methacrylate resin, sectioned and analysed. In paper I, we measured bone structure parameters such as trabecular number (Tb.N.), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), and trabecular bone volume (BV/TV) using bone histomorphometry.

Dual X-ray Absorptiometry (DXA)

DXA is a non-invasive method widely used for determining BMD in both animals and humans. It has the advantage of being able to distinguish between soft tissue and bone, made possible by the fact that different tissues absorb X-ray to different extents. An emitted X-ray is divided into two different energy levels by a filter. Sensors detect the amount of radiation absorbed when each X-ray passes through the body. Using different pre-set thresholds, bone tissue may be either included or excluded from the measurement. One important disadvantage when using DXA for determination of bone mass is that the images produced are only two-dimensional (2D). The DXA therefore only recognises changes in length and width and does not account for changes in the third dimension. This becomes a problem when examining growing animals with major skeletal changes in size. The areal bone mineral density (aBMD; g/cm^2) determined by DXA should not be mistaken for true volumetric BMD (vBMD; g/cm^3). In papers I and II, DXA measurements were performed with the Norland pDEXA Sabre (Norland, Fort Atkinson, Wisconsin, USA) and the Sabre Research software (v3.6) (73). In the *in vivo* measurements, one mouse was included in all the scans as an internal control, to avoid inter-scan variations. For *in vivo* measurements, medium resolution scans with the line spacing set at 500 μm were used, and for *ex vivo* measurements, high resolution scans were used (line spacing 100 μm). The inter-assay CV for the DXA measurements was less than 5%.

Peripheral Quantitative Computerised Tomography (pQCT)

pQCT is useful for the measurement of bone mass in animals and humans. However, the high radiation dose has limited its value in the clinical setting. pQCT is based on a rotating X-ray source, which moves to fixed positions around the specimen. A computer processes local attenuation data from each position and produces an image that represents a section through the specimen. The pQCT method admits measurements of cortical and trabecular

parameters of bone separately, which, in addition to measuring true vBMD, is an important advantage of this method. In papers I-III and V we measured two sections of the long bones; one in the mid-diaphysis, which consists only of cortical bone, and one in the metaphysis, consisting of both cortical and trabecular bone. Trabecular bone is defined as the inner 45% of the total cross-sectional area. The growth plate is used as a reference point in determining where to place the CT scan along the longitudinal axis. The pQCT measurements in this thesis were performed using the Stratec pQCT XCT Reasearch M (Version 4.5B,Norland, Fort Atkinson, Wisconsin, USA) operating at a resolution of 70 μm. The inter-assay CV for the pQCT measurements were less than 2%.

Micro-Computer Tomography (μCT)

While low bone mass is a major component of fracture risk, the bone micro-architecture, which defines the distribution of bone mass in three-dimensional space, also contributes to the biomechanical integrity of the tissue, and therefore also to fracture risk. μCT scanning makes it possible to analyse the architecture of bones at the trabecular level non-destructively, both qualitatively by visual inspection of informative three dimensional (3D) images of the trabecular network, and quantitatively by calculation of 3D morphometric parameters (111, 112). Bone studies are well suited for the μCT technique owing to the high contrast between calcified and soft tissue. The resolution with this technique is 5 μm.

The subject is placed on a rotating stage between an X-ray source and a charged-coupled detector (CCD) array. The spatial resolution of the image is primarily determined by the focal spot size of the X-ray source, the detector's array resolution, and the subject's position with respect to the source and detector. The imaging system provides a series of X-ray projections from a range of angles around the subject. Each projection represents the value of the X-ray attenuation line integral through the subject along the line from the X-ray source to the X-ray detector element. Imaging the subject at equiangular-spaced views over 180 degrees provides a complete set of projection data. Image reconstruction then creates a 2D image from the measured projection data and a 3D image is calculated by reconstructing and stacking individual 2D slices. Different algorithms are then used to calculate bone parameters such as bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), and trabecular number (Tb.N.). In papers II and III, we used a Skyscan 1072 scanner (Skyscan N.V.,

Aartselaar, Belgium). The X-ray tube voltage was 50 kV and the scanning angular rotation was 185°.

Mechanical testing

Bone mass measurements can be used to indicate bone quality, but mechanical testing can add more relevant data. In papers I and II we used three-point bending. The bone is placed horizontally on two supports and a force is directed vertically to the midshaft of the bone until it breaks. During the initial part of this process, the relationship between the force exerted on the material and the strain of the material will be linear. The slope of this linear relationship is the stiffness of the bone, also known as the elastic modulus or Young's modulus. Maximal stress is the maximum load under which the bone will break given in the unit force/area (Pascal). The force applied when the bone breaks is the maximum load, which is given in the unit Newton. Bone strength is not only dependent on the amount of mineral, but also on the external dimensions and the geometric arrangement of the mineralised mass. The area moment of inertia is a measurement of the radius from the cortex to the centre of the bone raised to the power of four. The importance of the area moment of inertia is illustrated by the greater cross-sectional area in the cortical bone seen in males as compared with females, which contributes to the sex difference in bone strength. In papers I and II, the bones were analysed using the Mechanical Tester 8841 (Instron, Canton, Massachusetts, USA). The bones were compressed at a constant speed of 2 mm/min until failure.

Analyses of gene expression

GeneChip DNA microarray

Microarray technology has opened new possibilities of large-scale mRNA expression profiling, where mRNA levels of many thousands of genes and expressed sequence tags (ESTs) can be analysed in one experiment, and requiring only a small amount of RNA. We used MG-U74Av2 Array (Affymetrix, Santa Clara, CA, USA) GeneChips. These GeneChip microarrays consist of small DNA probes, chemically synthesized at specific locations on a coated surface. Each gene is 25-mers and 16 different probes cover each gene. Each probe is synthesized to perfectly match the target gene (PM probe), and has a companion oligomer identical to the PM, except for a single base difference in a central position (the mismatch or MM probe). The MM probe serves as a control. The precise location where each probe is synthesized is

called a feature, and millions of features can be contained on one array. By extracting, amplifying, and labelling mRNAs from experimental samples and then hybridizing those prepared samples to the array, the amount of label can be monitored at each feature, enabling simultaneous relative quantification of the tens of thousands of different RNA transcripts, representing gene activity. The MG-U74Av2 Array consists of 6000 mouse genes and 6000 uncharacterised ESTs.

In paper IV, RNA was prepared from a homogenate of the humerus or the liver and pooled into two pools per animal group. The RNA was transcribed into cDNA and further into biotin-labelled cRNA, which was fragmented before hybridisation to the GeneChip. After washing, the chip was scanned and the output files were analysed using the Affymetrix Micro Array Suite Version 4.0.1 software. To allow for comparison of gene expression, the GeneChips were scaled to an average intensity of 500. Estrogen-regulated genes were determined by calculating the average fold change between vehicle-treated and estrogen-treated samples. Within each genotype, comparisons were then made between the two vehicle-treated and the two estrogen-treated GeneChips, generating a total of 4 comparisons for each genotype. The criteria for genes to be regarded as regulated by estrogen were as follows: (i) the absolute call for the gene had to be present for all GeneChips and the average difference had to be above 200, (ii) at least three of the four comparisons had to be considered increased or decreased according to Affymetrix algorithms, and (iii) the average fold increase or decrease of the four comparisons was to be at least 2.0-fold.

Real Time PCR

The polymerase chain reaction (PCR) is a very sensitive method for the quantification of specific mRNAs. The method is based on amplification of a cDNA sequence between a primer pair. In the real time PCR, the amplification can be followed over time and not only studied at the end of the reaction. A probe is designed to anneal between the two PCR primers. Two fluorescent dyes are attached to the 5′ and the 3′ends of the probe, a reporter and a quencher. When both dyes are attached to the probe, the reporter dye emission is quenched, but when the Taq DNA polymerase cleaves the reporter from the probe during the extension cycle, the reporter dye emits its characteristic fluorescence. The intensity of the emitted fluorescence is proportional to the amount of amplicon produced. Only specific hybridisation generates a fluorescent signal. Two different mRNAs

can be measured simultaneously in the same reaction tube as long as their probes are labelled with different fluorescent dyes for the reporters. This allows the use of an internal standard. In paper IV, real time PCR was used on individual mice to confirm regulation of genes regulated in the pooled samples used for the microarray analysis.

Results and comments

Papers I and II

Sex steroids are important not only to the female skeleton, but also to the male skeleton. Experimental and clinical data support the importance of androgen and estrogen in the maintenance of normal BMD in males; however, the relative contribution of androgen versus estrogen in the regulation of the male skeleton is unclear. In order to investigate the relative importance of ERα and ERβ in the regulation of adult bone metabolism in male mice, we used orx WT and ER-inactived ($ER\alpha^{-/-}$ (ERKO), $ER\beta^{-/-}$ (BERKO) and $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO)) male mice and treated them with 17β-estradiol or vehicle (paper I). Furthermore, to directly compare the effects of ER activation on bone with the effects of AR activation, orx WT and $ER\alpha^{-/-}$ $ER\beta^{-/-}$ mice were treated with 17β-estradiol, the non-aromatisable androgen DHT, or vehicle (paper II). Previous studies have shown that ERα-inactivated mice have raised levels of endogenous estrogen and testosterone, owing to disturbed feedback regulation (113, 114). This explains why we used orx mice in these studies.

In gonadally intact male mice, no major differences were found regarding the amounts of trabecular bone. Orx decreased the total body aBMD and the trabecular vBMD to the same extent in all four genotypes. Treatment with 17β-estradiol prevented this orx-induced bone loss in WT and $ER\beta^{-/-}$ (BERKO) mice, while no effects of 17β-estradiol were found in $ER\alpha^{-/-}$ (ERKO) or $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) mice. These pQCT measurement results were further confirmed by histomorphometric measurements and by μCT analyses, showing reductions in bone volume. Trabecular BMD was preserved in the gonadally intact $ER\alpha^{-/-}ER\beta^{-/-}$ (DERKO) mice in paper I, indicating that a testicular factor is of importance for the maintenance of trabecular bone mass. In paper II, we demonstrate that this testicular factor is androgens, which act on the AR. pQCT analyses showed that DHT treatment was as good as 17β-estradiol treatment in preventing the orx-induced trabecular bone loss in WT mice. Furthermore, μCT analyses demonstrated that 17β-estradiol treatment prevented the orx-induced reduction in bone volume as a result of both preserved number and preserved thickness of trabeculae in WT mice. DHT treatment only preserved the number of trabeculae, while trabeculae thickness was unaffected. Cortical bone parameters (BMC, area, and thickness) were increased after 17β-estradiol

treatment in orx ERα$^{+/+}$ mice, whereas DHT treatment had no effects on the cortical bone. (fig. 7).

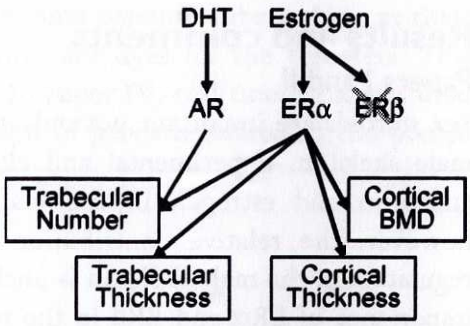

Fig. 7. Differential effects on bone of estrogen receptor (ER)α and androgen receptor (AR) activation in adult male mice.

It has been shown by others that estrogen treatment prevents orx-induced bone loss, but we demonstrate here for the first time that this effect of estrogen in male mice takes place exclusively via the ERα, while ERβ is of no importance. DHT and 17β-estradiol prevented orx-induced trabecular bone loss to the same extent; however, upon closer examination of the bone architecture, it became clear that AR and ER activation both preserved the number of trabeculae while the trabeculae thickness was exclusively preserved by ER activation. IGF-I serum levels were only affected by 17β-estradiol and not by DHT, and association studies showed that parameters that were affected only by 17β-estradiol and not by DHT (trabeculae thickness, cortical parameters and mechanical strength) did have a strong correlation to serum IGF-I levels.

Previous *in vitro* studies have indicated that there is cross-reactivity between ERs and AR (70). However, we show that *in vivo*, the bone-sparing effect of 17β-estradiol treatment is only via ERs and not via AR. Moreover, the AR activator DHT increased the amount of trabecular bone independent of ERs.

Paper III

As shown in paper II, both ERα activation and AR activation result in a bone-sparing effect, although in two distinct ways. One may therefore speculate that combined treatment with selective ER and AR modulators might be beneficial in the treatment of osteoporosis. In paper III, we investigated the effects of combined estrogen and androgen treatment on trabecular bone mass and longitudinal bone growth in ovx rats.

As expected, 17β-estradiol treatment prevented the ovx-induced trabecular bone loss as measured with both pQCT and μCT. DHT treatment also increased the bone mass as compared to that of vehicle-treated ovx rats, although it did not totally prevent ovx-induced bone loss. The combination

of 17β-estradiol and DHT treatment was additive, resulting in higher trabecular bone mass with the combination of 17β-estradiol and DHT treatment, than for 17β-estradiol alone. μCT analysis confirmed the finding from paper II that 17β-estradiol treatment increases both the thickness and number of trabeculae while DHT treatment only increases the number of trabeculae. Furthermore, the combination of 17β-estradiol and DHT treatment was additive regarding the number of trabeculae.

The ovx-induced increase of the length of the tibia was retarded by 17β-estradiol, whereas DHT treatment did not have any effects on bone length. However, DHT in combination with 17β-estradiol treatment counteracted the inhibitory effect of 17β-estradiol alone on bone length. 17β-Estradiol treatment reduced the width of the growth plate, which was associated with reductions in the number of proliferative and hypertrophic chondrocytes, and a reduction in the height of terminally differentiated hypertrophic chondrocytes. Addition of DHT to 17β-estradiol treatment fully counteracted the 17β-estradiol-induced reduction in height of hypertrophic chondrocytes and partially counteracted the inhibitory effect on the number of proliferative chondrocytes, whereas it did not affect the number of hypertrophic chondrocytes.

Paper III confirmed what has been shown before in male mice regarding the effects of 17β-estradiol and DHT treatment alone on bone parameters. Furthermore, the paper also revealed that a combination of 17β-estradiol and DHT treatment can have complex consequences. The paper shows that a combination of androgen and estrogen treatment can result in either additive or antagonistic effects depending on which bone parameter is studied. These findings suggest that combined treatment with selective ER modulators and selective AR modulators might be beneficial in the treatment of trabecular bone loss in patients with osteoporosis.

Paper IV

Previous *in vitro* data indicates that ERβ has the capacity to repress the transcriptional activity of ERα (36, 72). Furthermore, ERα and ERβ signal in opposite directions from an AP-1 site when complexed with estradiol (71). The role of ERβ as a dominant negative regulator of ERα has not been studied *in vivo*. In paper IV, using microarray analysis of the humerus and the liver, we investigated the interplay between ERα and ERβ in a global sense.

When studying the humerus, 95% of the genes that were increased by estrogen in ovx WT mice were also increased by estrogen in ERβ$^{-/-}$ (BERKO) mice. The average stimulatory effect of estrogen was 85% higher in ERβ$^{-/-}$ (BERKO) mice than in WT mice. When we looked at individual genes, we found that 80% of the genes were regulated more by estrogen in ERβ$^{-/-}$ (BERKO) than in WT mice. Similar results were found when looking at the gene expression pattern in the liver.

This study clearly shows that in the presence of ERα, ERβ reduces global estrogen-stimulated gene transcription in bone and liver, demonstrating that ERβ is a global inhibitor of ERα-regulated gene transcription in mice.

Paper V

Development of ER ligands that retain the beneficial effects of estrogen in the targeted tissue, e.g. bone, but lack mitogenic and carcinogenic activity in breast and uterus, remains a major challenge. SERMs are ER ligands which, in some tissues, act like estrogens, but which block estrogen action in others. ANGELS, on the other hand, are mechanism-specific ligands of ER. Estren has been proposed to increase bone mass via non-genotropic estrogen-like signaling without affecting reproductive organs or classic transcription (105). In Paper V, estren was further characterised both *in vivo* (tissue specificity) and *in vitro* (transcriptional activity).

In contrast to findings in a previous publication (105) estren treatment did have a clear effect on uterus weight in ovx mice compared to vehicle treatment. Thymus weight was decreased to the same extent by both 17β-estradiol and estren treatment. Regarding bone parameters, estren increased trabecular vBMD while there were no effects of estren treatment on any cortical bone parameter. A previous study has indicated that the effects of estren can be mediated via ERs and AR with the same efficiency (70). To investigate whether estren can act via AR *in vivo*, we treated ovx ERα$^{-/-}$ERβ$^{-/-}$ mice with estren. None of the effects of estren seen in ovx WT mice was found in these ER-inactivated mice. To further test the transcriptional activity of estren, human 293 kidney epithelial ERα- and ERβ reporter cells were treated with estren. Estren displayed a full agonistic effect on the transcriptional activity, similar to the effects of 17β-estradiol, although the affinity was lower. The results in this paper are in contrast with previous papers by Kousteni et al. (105). Here we show that estren actually has effects on the uterus, and that these effects are of the same order of magnitude as on trabecular vBMD. We also show that most of the effect of estren in ovx

female mice is mediated via ERs. Furthermore, estren has the capacity to exert classic ER mediated transcription.

Discussion

Importance of sex steroids for the male skeleton

Estrogen and testosterone have generally been considered the main sex steroids regulating bone metabolism in women and men, respectively. However, the discovery that several adult men who cannot produce or respond to estrogen are osteopenic, led to reconsideration of this notion (78-80). Recent longitudinal studies indicate, moreover, that bioavailable estrogen correlates better than testosterone with loss of BMD in elderly men (115, 116). Studies in rodents have also shown that estrogen treatment prevents orx-induced bone loss in male rodents (93, 99, 117). Both aromatase deficiency (118, 119) and aromatase inhibition (120, 121) in rodents lead to decreases in the amounts of trabecular bone, which can be prevented by estrogen treatment (119, 122). Today, it is generally accepted that estrogen is of importance for the vBMD of trabecular bone in elderly men (123). Using ER-inactivated mice, we studied the importance of ERα and ERβ for the maintenance of trabecular bone mass in male mice. In paper I, we clearly demonstrated that estrogen treatment only prevented orx-induced bone loss in presence of ERα, whereas presence of ERβ was of no importance. Estrogen treatment totally prevented the orx-induced bone loss in WT and $ER\beta^{-/-}$, while it had no effects on the trabecular bone mass in $ER\alpha^{-/-}$ or $ER\alpha^{-/-}ER\beta^{-/-}$ male mice.

It has previously been shown that androgen treatment and activation of AR can prevent orx-induced bone loss (93, 97, 99). In paper I, we speculate that acquisition of trabecular bone mass in the ERα-inactivated mice was attributable to activation of the AR by androgens. It was further demonstrated in paper II, that AR activation by the non-aromatisable DHT actually prevents the orx-induced bone loss in ERα-inactivated male mice. The quality of the bone should not only be regarded as determined by the trabecular BMD but also the micro-architecture of the bone (124). A closer look at the micro-architecture by use of μCT analysis revealed that while ERα activation maintained both the trabeculae thickness and number, AR activation only maintained the trabecular mass in terms of increased number of trabeculae. Furthermore, cortical bone was only preserved after orx by estrogen treatment and not by DHT treatment. This regulation of cortical bone and trabecular thickness by estrogen correlated well with serum levels of IGF-I. We and others have recently shown that endocrine, liver-derived IGF-I is an important regulator of the amount of cortical bone and its

mechanical strength (125, 126). We therefore propose that activation of ERα but not AR results in raised serum levels of IGF-I, which, in turn, increases the amount of cortical bone and the trabecular thickness in male mice (fig. 7). Together, ERα and AR are important for the maintenance of trabecular bone mass in male mice, whereas ERβ is of no importance.

Importance of sex steroids for the female skeleton

It is well established that estrogen is an important regulator of the female skeleton. Postmenopausal osteoporosis is the most common form of osteoporosis. Ovx of rodents has long been used as a model for osteoporosis and is associated with dramatic decreases in trabecular bone, which can be prevented by estrogen treatment (92, 107, 127). Administration of a selective ER antagonist, ICI 182,780, induces trabecular loss in intact and estrogen-repleted ovx female rats (128-130). Female mice with aromatase deficiency also have trabecular osteopenia, which can be prevented by estrogen treatment (98, 118, 119). Both estrogen acting on ERs and the non-aromatisable androgen DHT acting on AR have bone-sparing effects in ovx rats (131-133). The notion that androgens are also important to the female skeleton is further supported by the fact that hyperandrogenic women have increased trabecular BMD (134).

The combination of ER and AR activation in female rats was investigated in paper III. The study revealed that combined estrogen and androgen treatment results in an additive effect on the trabecular bone. As for the male mice, AR receptor stimulation increased the number of trabeculae, whereas ER stimulation increased both the number and thickness of trabeculae, which means that the additive effects of ER and AR activation on the bone mass were mainly attributable to an additive effect in terms of the number of trabeculae. These results contradict the findings in a previous study by Coxam et al. (133) where a combination of DHT and estrogen treatment did not alter the effects of single ER activation. This discrepancy between the two studies might be due to the difference in dose of DHT used or the difference in length between ovx and start of treatment. In the present study, treatment was initiated immediately after ovx, whereas in the previous study treatment only began after eight weeks.

Estrogen and androgens are also known to be important to longitudinal growth. Ovx of female rats increases longitudinal growth (96, 129, 130, 135, 136). In paper III, AR activation was found to counteract the inhibitory effect of ER activation on longitudinal bone growth, while it had no impact

on the estrogenic effect on the cortical bone. This disclosed that the results of combined AR and ER activation are highly complex, and depend on the parameters studied.

ERβ represses transcriptional activity of ERα

In contrast to the findings in male mice, ERβ is involved in the regulation of longitudinal growth in female mice. Female ERβ$^{-/-}$ mice have extended long bones, such that the normal sex differences regarding bone length is eliminated (73, 137). ERβ is also important to the closure of the growth plate in female mice (138). Thus, ERβ represses longitudinal growth in female mice and is suggested to be required for the sexual dimorphism of bone growth in mice. Both female and male ERα$^{-/-}$ mice have decreased longitudinal growth (137, 139, 140). Subsequently, ERα and ERβ have opposing effects with regard to bone growth in female mice. Furthermore, female ERβ$^{-/-}$ mice have increased cortical bone dimensions and are partially protected against the age-related trabecular bone loss that normally affects WT mice (74). As early as 1997, Paech et al. (71) demonstrated *in vitro* that ERα and ERβ can exert opposing effects in the modulation of gene expression. Opposing effects of ERα and ERβ have also been found in uterus and prostate (141, 142) and we have previously proposed that ERβ also exhibits an inhibitory action on ERα in thymus involution and fat reduction (143). In paper IV, we show for the first time *in vivo* that ERβ interacts with ERα on the transcriptional level. In the presence of ERα, ERβ is not required for the gene transcription. However, when ERβ is absent, the transcriptional activity of ERα is enhanced, demonstrating that ERβ is mainly a regulator of the ERα activity. Both the stimulatory and the inhibitory effects of ERα on gene transcription were less pronounced in the presence of ERβ. The study was performed in humerus and similar regulation was also found in liver, demonstrating that this is a global event and not specific to bone. However, in tissues such as prostate, ovary and lung tissue, it is clear that ERβ plays a major regulatory role in itself. It is important to take into account that ERβ exhibits an inhibitory action on ERα-mediated gene expression and in many instances opposes the actions of ERα when developing SERMs with an ERβ antagonist effect. The role of ERβ antagonists might be to enhance the effects of ERα activation more than being direct independent effects on the transcriptional machinery. In conclusion, this is the first time it has been shown *in vivo* that ERβ represses the ERα transcriptional activity.

Estren is not bone specific and it has transcriptional activity

Synthetic compounds that behave like estrogen in some tissues such as bone, while in others such as breast and uterus they block the estrogen activity are of great clinical relevance. The most well known SERMs are tamoxifen and raloxifene (144). Tamoxifen is an ER antagonist in the breast and it is available as endocrine therapy for postmenopausal women with ER-positive breast cancer. Raloxifene is mainly approved for treatment and prevention of osteoporosis. Like tamoxifen, raloxifene acts as an estrogen antagonist in breast tissue. Both tamoxifen and raloxifene are agonists in the uterus and liver. This results in increased incidences of uterine cancer and thromboembolic phenomena. The relative risk of venous thrombosis is increased two to three-fold with each of these medications (144, 145). Because of these side-effects, the development and improvement of new selective estrogen receptor modulators is an ongoing process.

Recently, it was proposed by Kousteni et al. (105) that the synthetic compound estren has non-genomic bone-sparing effects via stimulation of the Src/shc/extracellular-signal regulated kinase (ERK) and repression of the c-Jun N-terminal kinase (JNK) signaling cascades, leading to downstream modulation of the activity of transcription factors such as Elk-1, CCAAT/enhancer binding protein β (C/EBPβ), CREB, and c-Fos/c-Jun (70). These bone-sparing effects are clearly distinguished from the effects on the uterus, which is via the classic genomic pathway, with no effects of estren on the reproductive tissues. Therefore, estren has been suggested to be a mechanism-specific ligand of the ERs or the AR rather than being a tissue-specific ligand such as SERMs/SARMs. This finding, if accurate, is extremely important since one of the most crucial tasks in the development of new therapeutic agents for treatment of osteoporosis is to distinguish the bone-sparing effects from the effects on the reproductive tract. In our study, using a slightly lower dose of estren than that used by Kousteni et al., estren increased not only the trabecular BMD but also the uterine weight after ovx in female mice. The discrepancy between the findings in the two studies is difficult to explain, but it might be attributable to the differences in administration route. In the previous study, 60-day slow-release pellets were used. It might be possible that the delivery of estren was reduced during the last few days of the treatment period and therefore the effect on the uterus, which is a fast-responding tissue, was lost, while it remained in the more slow-responding bone tissue. In our study, daily injections were used for

administration, which ensured an unvarying dose over the whole treatment period.

The previously proposed lack of transcriptional activity for estren was explained after *in vitro* studies in ERα-transfected HeLa cells (70). However, the estrogenic effect in HeLa cells is exclusively dependent on AF-2, since these cells lack AF-1 (146, 147). In the 293 kidney epithelial ERα- and ERβ-expressing cells (148) used by us, estren had as good agonistic effect on the ERE-dependent transcriptional activity as 17β-estradiol, both in the presence of ERα and in the presence of ERβ. This demonstrates that the cell line selected for the experiment and its specific cellular environment is of great importance for the activity of estren. It was further confirmed that estren actually has classic transcriptional activity by use of the ER antagonist ICI 182,780 which, in a dose-dependent manner, antagonised the effects of estren on the transcription machinery. Although these results do not exclude non-genomic effects of estren, they clearly demonstrate, in contrast to previous findings, that estren has transcriptional activity.

Estren has also been proposed to act via both the ERs and the AR (70, 105). Cross-reactivity between the ERs and AR for mediation of the bone-sparing effects has also been proposed for the natural ligands for the receptors (70). However, as early as in papers I and II, we showed that the bone-sparing effect on the trabecular bone of 17β-estradiol and DHT is dependent on ERα and AR, respectively, while no cross-reactivity between the two receptors was found. In the present study, we show that ERs were required for the action of estren on the trabecular bone since no effect was found after estren treatment in the ovx double ER-inactivated mice. However, we have shown that estren inhibits T lymphopoiesis via ER-independent pathways, whereas its suppression of peripheral immune functions is ER-dependent (149). We have also demonstrated that estren is able to signal through the AR since estren has similar effects as DHT on lymphopoiesis in thymus and bone marrow, and on submandibular glands (150). These effects are all independent of the estrogen receptors since they were all present in double ER-inactivated female mice. It has also been demonstrated in male mice that estren has transcriptional activity via the AR (151). Taken together, these data show that estren has the capacity to activate the classic genomic transcriptional machinery via ERα, ERβ, and AR, although its activity is regulated differently in different tissues. Since it is

clear that estren is not bone-specific but also affects the reproductive organs, it is no longer a promising candidate for treatment of osteoporosis.

Conclusions

In this thesis, we demonstrate that both ERα and AR activation in adult male mice prevent orx-induced trabecular BMD loss, whereas ERβ is of no importance. ERα and AR activation increase the trabecular BMD by two distinct mechanisms since ERα activation increases both the thickness and number of the trabeculae while AR activation increases the number of the trabeculae but not the thickness. Furthermore, the bone parameters affected only by ERα correlate with serum levels of IGF-I, indicating that the effects mediated exclusively by ERα are mediated via IGF-I. We also show in ovariectomized female rats that a combination of ERα and AR activation has an additive effect on the trabecular BMD. In addition, we demonstrate for the first time *in vivo* that ERβ can function as a repressor of ERα mediated gene transcription. The notion that ERβ exhibits an inhibitory effect on ERα-mediated gene expression and in many instances opposes the actions of ERα must be taken into account when developing SERMs with an ERβ antagonist effect. The role of ERβ antagonists might be to enhance the effects of ERα activation more than to have direct effects on the transcriptional machinery. In conclusion, since the bone-sparing effects of ERα and AR activation in adult male mice are clearly distinct from each other, a combination of SERM and SARM might be beneficial in the treatment of osteoporosis.

Acknowledgements

I wish to express my sincere gratitude to everyone who has contributed to this thesis. Special thanks to:

Claes Ohlsson, the most excellent supervisor I can think of, always full of never-ending enthusiasm and positive thinking. Thank you for believing in me from the very beginning and for always having time whenever I needed it.

Olle Isaksson for providing good research facilities at RCEM.

All my co-authors. Special thanks to Katrien Venken for being such a nice and helpful person to collaborate with. Phil Salmon for teaching me the μCT technique and for answering my sometimes very simple questions regarding this lovely technique.

All my friends in the bone research group: Marie for introducing me to the bone field, and especially the animal work. Thank you for lighting up the days and sharing all your knowledge with me. Anette, for all your help with everything whenever it was needed. Anna-Lena E, Anna-Lena J, Charlotte, Jenny, Johan, Klara, Liesbeth, Lotta, Mattias, Maud, Niklas, and Sara, for making such a good atmosphere in the group.

My friends and colleagues at RCEM and brosklab. A special thank to Åsa for fun and fruitful discussions not only regarding science. Inger for being a good office mate.

Ulla-Britt, Maria and Anna-Lena for administrative help.

Per and Mathias for computer support.

Ann, Anna, Karin, Carl-Fredrik, Fredrik, and Thomas, for sharing the first really fun years at university with me. A special thanks to Ann, who taught me the basics of the real time PCR technique and for being my backup for everything I always forgot to order.

Helena O, Helena W, Linda, and Åsa, for making my first contact with science such a pleasant experience that it made me to continue.

Annika, svärmor, för att du har underlättat vardagen avsevärt.

Mamma, pappa och Andreas. Hjärtligt tack för att ni alltid har stöttat mig och för all hjälp under slutförandet av avhandlingen.

Maja, min lilla gullhöna, för att du finns och ger liv åt dagarna och så mycket att glädjas åt. Stanko, min älskade, för att du alltid har stöttat mig på ditt alldeles egna lilla sätt. Tack för att jag får dela livet med dig. ♥♥♥

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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 4, 2002

Two Different Pathways for the Maintenance of Trabecular Bone in Adult Male Mice

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ABSTRACT

Androgens may regulate the male skeleton either directly via activation of the androgen receptor (AR) or indirectly via aromatization of androgens into estrogen and, thereafter, via activation of estrogen receptors (ERs). There are two known estrogen receptors, ER-α and ER-β. The aim of this study was to investigate the relative roles of ER-α, ER-β, and AR in the maintenance of trabecular bone in male mice. Seven-month-old male mice, lacking ER-α (ERKO), ER-β (BERKO), or both receptors (DERKO), were orchidectomized (orx) and treated for 3 weeks with 0.7 μg/mouse per day of 17β-estradiol or vehicle. No reduction in trabecular bone mineral density (BMD) was seen in ERKO, BERKO, or DERKO mice before orx, showing that neither ER-α nor ER-β is required for the maintenance of a normal trabecular BMD in male mice. After orx, there was a pronounced decrease in trabecular BMD, similar for all groups, resulting in equal levels of trabecular BMD in all genotypes. This reduction was reversed completely in wild-type (WT) and BERKO mice treated with estrogen, and no significant effect of estrogen was found in ERKO or DERKO mice. In summary, the trabecular bone is preserved both by a testicular factor, presumably testosterone acting via AR and by an estrogen-induced activation of ER-α. These results indicate that AR and ER-α are redundant in the maintenance of the trabecular bone in male mice. In contrast, ER-β is of no importance for the regulation of trabecular bone in male mice. (J Bone Miner Res 2002;17:555–562)

Key words: estrogen receptors, androgens, bone, transgenic, males

INTRODUCTION

Morbidity from osteoporosis in the aging population is substantial in both men and women. However, few studies have been designed for the elucidation of the mechanism(s) behind male osteoporosis. It is obvious that androgens are important both for the acquisition of bone during skeletal growth and for the maintenance of trabecular bone in adults. The effects of testosterone on the skeleton can be exerted either directly via the androgen receptor (AR) or indirectly via aromatization to estrogen and further via estrogen receptors (ERs). Orchidectomy (orx) results in bone loss, which is prevented by treatment with androgens.[1,2] However, estrogen treatment also prevents orx-induced bone loss.[1,2] Humans, as well as rodents, with impaired aromatase activity, suffer from decreased bone mineral density (BMD),[3–8] which can be prevented by treatment with estrogen.[3,5,9,10] Furthermore, several stud-

The authors have no conflict of interest.

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ies have presented stronger correlations between BMD and estrogen than with testosterone.[11–13] These experimental and clinical data support the importance of estrogen in the maintenance of normal BMD in males. There are two known ERs, denoted α (ER-α) and β (ER-β). Previously, we have shown that ER-α, but not ER-β, mediates important effects of estrogen in the regulation of skeletal growth in male mice.[14] However, the relative importance of ER-α and ER-β in the regulation of adult bone metabolism in male mice is unknown. The lack of ER-α in male mice results in elevated serum levels of estrogen and testosterone, probably because of disturbed feedback regulations.[15] Therefore, to avoid confounding effects of elevated sex steroids, the ER-inactivated and wild-type (WT) mice used in this study were orx. The mice were substituted with 17β-estradiol or vehicle and the ER specificity in the regulation of the adult bone metabolism in males was investigated.

MATERIALS AND METHODS

Animals

Male double heterozygous (ER-$\alpha^{+/-}\beta^{+/-}$) mice were mated with female double heterozygous (ER-$\alpha^{+/-}\beta^{+/-}$) mice, resulting in WT, ER-$\alpha^{-/-}$ER-$\beta^{+/+}$ (ERKO), ER-$\alpha^{+/+}$ER-$\beta^{-/-}$ (BERKO) and ER-$\alpha^{-/-}$ER-$\beta^{-/-}$ (DERKO) offspring with a mixed C57BL/6J/129 background.[16,17] Genotyping of tail DNA was performed at 3 weeks of age as previously described.[14] 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). All mice were orx at the age of 7 months. The mice were left to recover for 10 days after orx. After recovery, mice were injected subcutaneously (sc) with 0.7 μg/mouse per day of 17β-estradiol benzoate (Sigma, St. Louis, MO, USA) for 5 days/week during 3 weeks. Control mice received injections of vehicle oil (olive oil; Apoteksbolaget, Göteborg, Sweden). Treatment with 0.7 μg/mouse per day of 17β-estradiol benzoate resulted in 17β-estradiol levels of ~220 pmol/liter, which could be regarded as physiologically relevant concentrations of 17β-estradiol.

Dual X-ray absorptiometry

Measurement of bone mineral content (BMC) and areal BMD (aBMD) of total body in vivo and femur in vitro was performed with the Norland Medical Systems pDEXA Sabre (Norland Medical Systems, Fort Atkinson, WI, USA) and the Sabre Research software (v3.6; Norland Medical Systems, Fort Atkinson, WI, USA) as previously described.[14,18]

Peripheral quantitative computerized tomography

Computerized tomography (CT) was performed with the Stratec pQCT XCT Research M (v5.4B; Norland Medical Systems) operating at a resolution of 70 μm as previously described.[18] Cortical parameters were determined with a middiaphyseal peripheral quantitative CT (pQCT) scan of the tibias. Trabecular BMD was determined with a metaphyseal pQCT scan of the proximal tibias and defined as the inner 45% of the total area.

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. Histomorphometric parameters measured were trabecular bone volume (BV/TV; %), trabecular thickness (Tb.Th; μm), trabecular separation (Tb.Sp; μm), and trabecular number (Tb.N; mm^{-1}).[19]

Mechanical testing

After pQCT measurements, the humerus was applied to mechanical testing using Mechanical Tester 8841 (Instron, Canton, MA, USA). Three-point bending force was measured by placing the bone horizontally with the anterior surface upward and applying pressing force vertically to the midshaft of the bone. Each bone was compressed with a constant speed of 2 mm/minute until failure. Breaking force (maximal load) was defined as bending load at failure. Maximal stress (sigma) and elastic or Young's modulus (E) were calculated as previously described.[20]

Radioimmunoassay

Serum insulin-like growth factor (IGF) I levels were measured by double antibody IGF binding protein–blocked radioimmunoassay (RIA).[21] Serum osteocalcin levels were measured using a monoclonal antibody raised against human osteocalcin (Rat-MID osteocalcin ELISA; Osteometer Biotech A/S, Herlev, Denmark). The sensitivity of the osteocalcin assay was 21.1 ng/ml and intra- and interassay coefficients of variation (CVs) were <10%. Levels of c-telopeptide were measured in serum by ELISA, which measures degradation products of type I collagen, generated by osteoclastic bone resorption.[22] The sensitivity of the ELISA was <0.1 ng/ml. The average intra- and interassay CVs were <12%. 17β-estradiol was measured using an RIA detecting estradiol (DiaSorin, Saluggia, Italy) with sensitivity below 5 pg/ml at 95% confidence limit.

Tartrate-resistant acid phosphatase 5b activity

Tartrate-resistant acid phosphatase (TRAP) 5b, purified from human osteoclasts as described,[23] was used as antigen to develop a polyclonal antiserum in rabbits.[24] The antiserum was incubated on anti-rabbit immunoglobulin G (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/liter of 4-nitrophenylphosphate (4-NPP) as substrate in 0.1 M of sodium acetate buffer, pH 6.1,

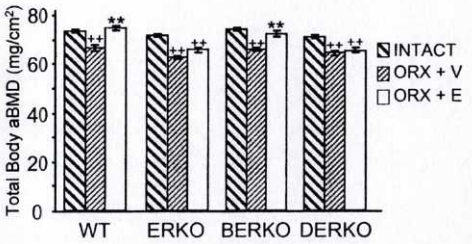

FIG. 1. Total body aBMD measured using DXA. Seven-month-old male mice were orx and treated with vehicle (V) or 17β-estradiol (E) for 3 weeks (intact animals [$n = 14$], orx animals [$n = 4-6$]). Values are given as means ± SEM; $^{++}p < 0.01$ versus intact mice and $^{**}p < 0.01$ versus vehicle. Student's t-test.

for 2 h at 37°C. The enzyme reactions were terminated by adding 25 μl of 0.32 M of NaOH, and A405 was measured using Victor² equipment (EG & G Wallac).

RESULTS

aBMD and BMC as determined by dual-energy X-ray absorptiometry

In vivo analysis of the total body aBMD in 7-month-old intact male mice revealed a slight decrease in ERKO (−2.7%) and DERKO (−3.6%) but not in BERKO compared with WT mice ($p < 0.05$; one-way analysis of variance [ANOVA], followed by Student-Newman-Keul's multiple range test; Fig. 1). After orx, the total body aBMD decreased to a similar extent in all four genotypes. Treatment with 17β-estradiol increased total body aBMD in WT and BERKO as compared with vehicle treatment, whereas no estrogenic effect was found in ERKO or DERKO mice (Fig. 1). After death the left femur was excised and analyzed by dual-energy X-ray absorptiometry (DXA) in vitro. Treatment with 17β-estradiol increased the femoral aBMD in WT and BERKO, whereas no effect was seen in ERKO or DERKO mice (Fig. 2A). The estrogen-induced increase in aBMD in WT and BERKO mice resulted in a similar increase in femur BMC (Fig. 2B). Thus, the effect of 17β-estradiol on femoral BMC in orx mice is ER-α–mediated. In contrast, no effect of treatment with 17β-estradiol was seen on the femur area (Fig. 2C).

The results obtained from DXA measurements are a combination of effects on trabecular bone and cortical bone parameters. To be able to distinguish between effects of 17β-estradiol on trabecular and cortical bone, the mice were analyzed by pQCT.

Trabecular BMD as determined by pQCT

The trabecular volumetric BMD (tvBMD) was measured in the metaphyseal region of the proximal tibias using pQCT. Measurements of tvBMD before orx revealed a slight increase in ERKO and DERKO ($p < 0.01$; one-way ANOVA, followed by Student-Newman-Keul's multiple range test), whereas no difference was seen in BERKO compared with WT mice (Figs. 3A and 3B). After orx, the tvBMD decreased dramatically to the same level in all genotypes. Treatment with 17β-estradiol prevented this decrease in WT and BERKO mice, whereas no effect of 17β-estradiol was seen in ERKO or DERKO mice (Figs. 3A and 3B).

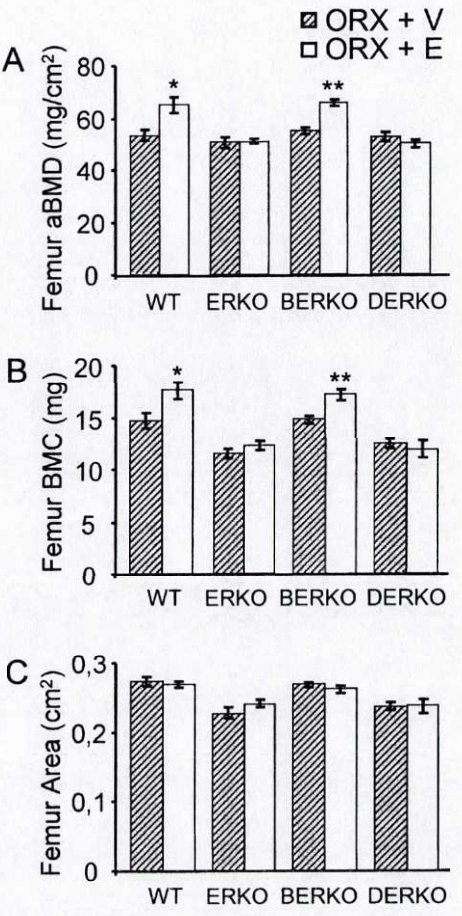

FIG. 2. Femur (A) aBMD (B) BMC, and (C) area measured using DXA. Seven-month-old male mice were orx and treated with vehicle (V) or 17β-estradiol (E) for 3 weeks ($n = 4-6$). Values are given as means ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle. Student's t-test.

Histomorphometry

Histomorphometry of the metaphyseal part of the proximal tibias was performed to confirm the effects on trabec-

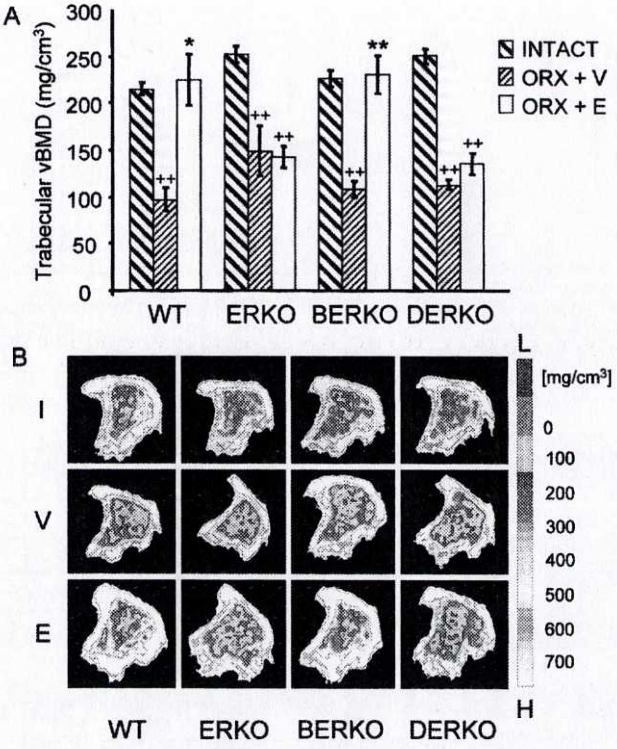

FIG. 3. (A) tvBMD of tibias measured using pQCT. (B) Representative pQCT scans. Seven-month-old intact (I) male mice were orx and treated with either vehicle (V) or 17β-estradiol (E) for 3 weeks. Intact animals (n = 14) and orx animals (n = 4–6). Values are given as means ± SEM, $^{++}p < 0.01$ versus intact mice, $^{*}p < 0.05$, and $^{**}p < 0.01$ versus vehicle, Student's t-test. H, high bone mineral density; L, low bone mineral density.

ular bone as detected by pQCT. Trabecular bone volume as a ratio to total bone volume (BV/TV) was well correlated to tvBMD as measured using pQCT ($R = 0.76$; $p = 3.1 \times 10^{-8}$). Estrogen treatment increased the BV/TV in WT. Statistical analysis in which ER-$\alpha^{+/+}$ (WT and BERKO) and ER-$\alpha^{-/-}$ (ERKO and DERKO) mice were analyzed independently indicated that 17β-estradiol treatment increased BV/TV in ER-$\alpha^{+/+}$ animals but not in ER-$\alpha^{-/-}$ animals ($p < 0.01$; two-way ANOVA, followed by Student-Newman-Keul's multiple range test; Table 1), confirming our results of the effect of 17β-estradiol on tvBMD as measured using pQCT. Tb.Sp was decreased and Tb.N was increased after estrogen treatment in WT and BERKO mice, whereas no effect was seen in ERKO or DERKO mice (Table 1).

Cortical bone parameters (pQCT)

Cortical bone parameters (BMC, area, and thickness) were measured by a middiaphyseal pQCT scan of the tibias. These cortical bone parameters were decreased in 7-month-old intact ERKO and DERKO but not in BERKO mice compared with WT mice (data not shown). Cortical BMC was increased after estrogen treatment in WT and BERKO but not in ERKO or DERKO mice (Table 2). Similar tendencies were seen regarding both cortical thickness and cross-sectional area (Table 2). Statistical analysis in which ER-$\alpha^{+/+}$ (WT and BERKO) and ER-$\alpha^{-/-}$ (ERKO and DERKO) mice were analyzed independently showed that 17β-estradiol treatment increased all these cortical bone parameters in orx ER-$\alpha^{+/+}$ (BMC, 24 ± 4% and $p < 0.01$; thickness, 16 ± 3% and $p < 0.01$; cross-sectional area, 19 ± 3% over vehicle and $p < 0.01$; two-way ANOVA, followed by Student-Newman-Keul's multiple range test), whereas no effect was seen in ER-$\alpha^{-/-}$ mice.

Mechanical strength

The mechanical strength was measured using three-point bending of the humerus. Maximal load, a measurement of the strength of the bone, was increased after 17β-estradiol treatment in orx ER-$\alpha^{+/+}$ (WT and BERKO) mice (28 ± 8% over vehicle and $p < 0.01$ two-way ANOVA, followed by Student-Newman-Keul's multiple range test). No effect of 17β-estradiol treatment was seen in ER-$\alpha^{-/-}$ (ERKO and DERKO) mice, showing an ER-α–mediated effect. The qualitative bone parameter maximal stress and elastic mod-

TABLE 1. HISTOMORPHOMETRIC PARAMETERS

		WT	*ERKO*	*BERKO*	*DERKO*
BV/TV (%)	V	3.0 ± 0.8	4.7 ± 0.5	5.6 ± 0.8	5.2 ± 1.1
	E	11.7 ± 3.3*	6.0 ± 1.3	11.9 ± 2.8‡	6.0 ± 1.2
Tb.Sp (μm)	V	983 ± 206	545 ± 55	613 ± 71	684 ± 106
	E	419 ± 106*	565 ± 119	347 ± 83*	481 ± 50
Tb.N (mm^{-1})	V	1.1 ± 0.2	1.8 ± 1.2	1.6 ± 0.9	1.5 ± 0.3
	E	2.6 ± 0.7*	1.9 ± 0.4	3.2 ± 0.6*	2.0 ± 0.2
Tb.Th (μm)	V	26.8 ± 3.5	25.9 ± 2.9	34.0 ± 4.2	36.5 ± 10.8
	E	44.0 ± 2.7†	31.1 ± 1.7	36.5 ± 5.7	28.9 ± 2.6

Histomorphometric analysis was performed on proximal tibia from orx mice treated with vehicle (V) or 17β-estradiol (E) for 3 weeks. BV/TV, Tb.Sp, Tb.N, and Tb.Th were measured. Values are given as means ± SEM (n = 4–6).
*$p < 0.05$; †$p < 0.01$; ‡$p = 0.056$ versus vehicle, Student's t-test.

TABLE 2. CORTICAL BONE PARAMETERS

		WT	*ERKO*	*BERKO*	*DERKO*
BMC (mg/mm)	V	0.89 ± 0.04	0.81 ± 0.06	0.87 ± 0.02	0.85 ± 0.03
	E	1.08 ± 0.07*	0.88 ± 0.05	1.09 ± 0.04†	0.80 ± 0.03
Thickness (μm)	V	185 ± 10	179 ± 8	183 ± 4	191 ± 5
	E	214 ± 12	201 ± 8	214 ± 5†	183 ± 7
Cross-sectional area (mm^2)	V	0.79 ± 0.02	0.73 ± 0.05	0.80 ± 0.02	0.77 ± 0.02
	E	0.94 ± 0.06‡	0.79 ± 0.05	0.94 ± 0.03†	0.72 ± 0.03

Cortical bone parameters were determined using a middiaphyseal pQCT scan of the tibia from orx mice treated with vehicle (V) or 17β-estradiol (E). Values are given as means ± SEM (n = 4–6).
*$p < 0.05$; †$p < 0.01$; ‡$p = 0.053$ versus vehicle, Student's t-test.

TABLE 3. SERUM PARAMETERS

		WT	*ERKO*	*BERKO*	*DERKO*
Osteocalcin (ng/ml)	V	88.0 ± 4.9	162.6 ± 47.1	133.3 ± 7.2	130.3 ± 22.5
	E	114.9 ± 36.3	150.7 ± 22.1	111.4 ± 14.7	142.5 ± 10.6
c-Telopeptide (ng/ml)	V	14.7 ± 1.4	10.9 ± 0.9	10.8 ± 0.3	8.7 ± 1.0
	E	10.6 ± 0.8*	8.8 ± 1.1	10.4 ± 1.4	11.2 ± 0.9
TRAP 5b (U/liter)	V	3.0 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	2.8 ± 0.4
	E	5.3 ± 0.7*	3.1 ± 0.4	5.2 ± 0.8†	3.2 ± 0.2
IGF-I (ng/ml)	V	210.6 ± 27.7	222.6 ± 21.6	284.8 ± 25.9	270.5 ± 32.6
	E	319.7 ± 44.7	248.4 ± 21.1	286.9 ± 17.4	265.3 ± 28.3

Serum was collected from orx mice treated with vehicle (V) or 17β-estradiol (E) for 3 weeks. Values are given as means ± SEM (n = 4–6).
*$p < 0.05$; †$p < 0.01$ versus vehicle, Student's t-test.

ulus were not affected by 17β-estradiol treatment in any genotype (data not shown).

Serum levels of biochemical bone parameters and IGF-I

Serum parameters were measured at death after 3 weeks of treatment with estrogen or vehicle to orx mice. No major effects of 17β-estradiol treatment were seen on the serum levels of osteocalcin or c-telopeptide (Table 3). The activity of TRAP 5b, an osteoclast-specific enzyme, was increased after 17β-estradiol treatment in WT and BERKO mice, whereas no effect was detected in ERKO or DERKO mice as compared with vehicle treatment (Table 3). The activity of TRAP 5b was correlated to tvBMD, as determined using pQCT ($R = 0.76$; $p = 4.4 \times 10^{-8}$) as well as BV/TV, determined by histomorphometry ($R = 0.75$; $p = 1.1 \times 10^{-7}$). Serum levels of IGF-I were not significantly affected by estrogen treatment in any of the genotypes (Table 3).

DISCUSSION

Estrogen is of importance for the regulation of skeletal growth and maturation in female mice and an increasing

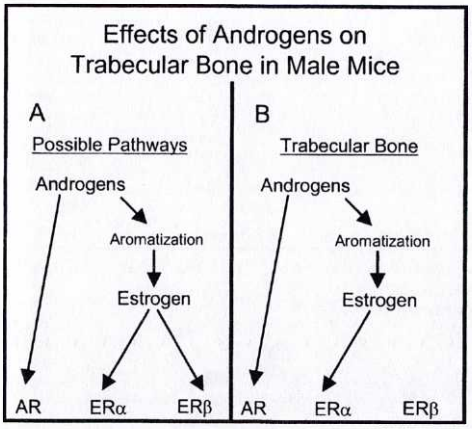

FIG. 4. Effects of androgens on trabecular bone in male mice. (A) Three different possible pathways for androgens in the regulation of trabecular bone in male mice are indicated. (B) Our present data together with some previous findings show that the trabecular bone is preserved both by an activation of ER-α and by an activation of the AR.[1,25] Thus, we propose that the AR and ER-α are redundant in the regulation of trabecular bone in male mice. In contrast, ER-β is of no importance for the regulation of trabecular bone in male mice.

amount of data indicates that estrogen is of importance in male mice as well. Previously, we have shown that the skeletal growth of the long bones in male mice is dependent on estrogen, because combined loss of both ER-α and ER-β results in impaired longitudinal bone growth. This phenotype also was seen in male ERKO, but not in male BERKO mice, showing an ER-α–mediated effect.[14] Male aromatase deficient mice (ArKO) have shorter femurs than their normal littermates (NLMs), further supporting the importance of estrogen in the regulation of longitudinal bone growth in male mice.[6] Interestingly, androgens acting directly on the AR were not able to substitute for the loss of ER-α in the stimulation of longitudinal bone growth in male mice.[14] Thus, ER-α and AR are not redundant regarding the regulation of longitudinal bone growth in male mice.

Our previous study, analyzing ER-inactivated young adult male mice with intact gonads, did not detect any major effect on the amount of trabecular bone. However, these ER-inactivated mice have increased levels of endogenous estrogen and testosterone, which might have confounded the results on the amount of trabecular bone.[15,25] Therefore, to avoid confounding effects of elevated sex steroids, the ER-inactivated and WT mice used in this study were orx. Androgens may regulate the amount of trabecular bone either directly via activation of AR or indirectly via aromatization into estrogens and further via activation of ERs (Fig. 4A). A protective role of estrogen on trabecular bone is supported by the fact that aromatase deficiency in male mice[6,9] as well as aromatase inhibition in male rats[7,8] results in a slight decrease in the amount of trabecular bone. Treatment with estrogen prevents this bone loss.[9,10] This study confirms some previous studies by showing that orx-induced trabecular bone loss is prevented by estrogen treatment.[1,2] However, for the first time, we have elucidated the ER specificity for this bone-sparing effect of estrogen in male mice. 17β-Estradiol treatment of orx male mice prevents trabecular bone loss in WT and BERKO but not in ERKO or DERKO mice. This finding clearly shows that the protective effect of estrogen on trabecular bone in adult male mice is mediated via ER-α, and ER-β is of no importance.

The orx of adult male rats causes trabecular bone loss that can be prevented both by testosterone and by nonaromatizable androgen dihydrotestosterone (DHT).[1] These data show a protective role of androgens on the trabecular bone via activation of AR (Fig. 4B). In this study, the physiological role of AR in the regulation of trabecular bone was analyzed indirectly. The fact that the trabecular BMD is preserved in ER-α as well as in ER-α/β double-inactivated male mice with intact gonads indicates that a testicular factor, presumably testosterone acting via the AR, is able to maintain a normal trabecular BMD. This notion is supported by a recent study in which testosterone prevented orx-induced bone loss in ERKO mice.[25] Furthermore, the orx-induced trabecular bone loss in this study is similar in WT and DERKO mice. Thus, a testicular factor is able to maintain the trabecular bone in male mice devoid of all known ERs. Because a similar magnitude of orx-induced trabecular bone loss is seen in mice having functional ERs and in mice devoid of all known ERs, this testicular factor is most likely acting via the AR. Previous studies indicate that the serum levels of testosterone in orx rats are 5–10% of that in intact rats.[7] Therefore, one cannot exclude that a low background concentration of adrenal-derived androgens may have reduced the effect of removal of testicular androgens. Our present data together with previous findings[1,25] show that trabecular bone is preserved both by an activation of ER-α and by an activation of the AR (Fig. 4B). Neither male mice with ER-α inactivation nor rats with a nonfunctional AR suffer from any major trabecular bone loss.[26] Thus, we propose that the AR and ER-α are "redundant" in the regulation of trabecular bone in male rodents (Fig. 4B). In contrast, as discussed previously, no redundancy between ER-α and AR was seen for the regulation of longitudinal bone growth in male mice.

A recent in vitro study indicated that both androgens and estrogens are able to exert nongenomic effects via activation of either the AR or ERs.[27] We could not detect any effect of estrogen on the trabecular bone in orx male DERKO mice, suggesting that an in vivo effect of estrogen via the AR is unlikely. However, our study does not rule out the possibility that androgens might be able to exert effects via ERs. Treatment of orx AR-deficient mice with nonaromatizable DHT could test this possibility.

Young adult ERKO and DERKO but not BERKO mice with intact gonads have decreased cortical BMC compared with WT mice, showing that the estrogenic regulation of cortical BMC is mediated via ER-α.[14] Treatment of adult orx mice with estrogen resulted, in this study, in a small but significant increase in the cortical BMC in WT and BERKO male, whereas no effect of estrogen treatment was found in

ERKO or DERKO mice. In addition, these data show that the effects of estrogen on adult cortical BMC are mediated via ER-α and not ER-β. The effects on cortical BMC were reflected by similar changes in mechanical strength as measured by three-point bending. Estrogen increased the maximal load in ER-$\alpha^{+/+}$ but not in ER-$\alpha^{-/-}$ mice. However, there was no effect on qualitative bone parameters, including elastic modulus and maximal stress, indicating that the quality of the cortical bone is not affected by estrogen treatment.

Analysis of total body areal BMD using DXA revealed a small decrease in intact ERKO and DERKO mice compared with WT mice. The image produced by the DXA is two-dimensional and does not recognize changes in the third dimension. Therefore, the reduction in areal BMD seen in male ERKO and DERKO mice with intact gonads probably is caused by decreased size of the animals and does not reflect a true decrease in vBMD.

Serum levels of osteocalcin, a marker of bone formation, and *c*-telopeptide, a marker of bone resorption, were measured at the end of the study after 3 weeks of treatment with estrogen. No major effect of estrogen was detected for these markers in any genotype. On the contrary, the recently described new bone resorption marker, serum TRAP 5b activity,[28,29] was increased after estrogen treatment in WT and BERKO mice whereas the activity was unaffected by estrogen treatment in ERKO and DERKO mice. It is well known that the acute effect of estrogen in gonadectomized rodents is to decrease TRAP 5b.[30] In contrast, in this study we have found that TRAP 5b activity is increased after a prolonged estrogen treatment. Interestingly, the increased TRAP 5b levels were correlated with the amount of trabecular bone. Similarly, recently, we reported that long-term orx results in a decreased TRAP 5b activity, associated with a decrease in trabecular BMD.[30] Thus, one might speculate that TRAP5b reflects the amount of trabecular bone after a prolonged estrogen treatment. In summary, the trabecular bone is preserved both by a testicular factor, presumably testosterone acting via AR, and by an estrogen-induced activation of ER-α. These results indicate that AR and ER-α are redundant in the maintenance of the trabecular bone in male mice (Fig. 4B). In contrast, ER-β is of no importance for the regulation of trabecular bone in male mice.

ACKNOWLEDGMENTS

We thank Anette Hansevi and Maud Pettersson for valuable technical assistance. We also thank SWEGENE Center for Bio-Imaging (CBI), Gothenburg University, for technical support regarding image analysis. This study was supported by the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, the Lundberg Foundation, the Torsten and Ragnar Söderbergs Foundation, the Emil and Vera Cornell Foundation, Petrus and Augusta Hedlunds Foundation, Novo Nordisk Foundation, the Swedish Association Against Rheumatic Disease, the Swedish Cancer Fund, the National Institutes of Health (NIH) grant support AR31062, and Karo Bio AB.

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Received in original form July 16, 2001; in revised form September 25, 2001; accepted October 18, 2001.

II

Differential effects on bone of estrogen receptor α and androgen receptor activation in orchidectomized adult male mice

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Edited by Elwood V. Jensen, University of Cincinnati Medical Center, Cincinnati, OH, and approved September 5, 2003 (received for review May 22, 2003)

Androgens may regulate the male skeleton either directly by stimulation of the androgen receptor (AR) or indirectly by aromatization of androgens into estrogens and, thereafter, by stimulation of the estrogen receptors (ERs). To directly compare the effect of ER activation on bone *in vivo* with the effect of AR activation, 9-month-old orchidectomized wild-type and ER-inactivated mice were treated with the nonaromatizable androgen 5α-dihydrotestosterone, 17β-estradiol, or vehicle. Both ERα and AR but not ERβ activation preserved the amount of trabecular bone. ERα activation resulted both in a preserved thickness and number of trabeculae. In contrast, AR activation exclusively preserved the number of trabeculae, whereas the thickness of the trabeculae was unaffected. Furthermore, the effects of 17β-estradiol could not be mediated by the AR, and the effects of 5α-dihydrotestosterone were increased rather than decreased in ER-inactivated mice. ERα, but not AR or ERβ, activation resulted in preserved thickness, volumetric density, and mechanical strength of the cortical bone. ERα activation increased serum levels of insulin-like growth factor I, which were positively correlated with all the cortical and trabecular bone parameters that were specifically preserved by ERα activation but not by AR activation, suggesting that insulin-like growth factor I might mediate these effects of ERα activation. Thus, the *in vivo* bone-sparing effect of ERα activation is distinct from the bone-sparing effect of AR activation in adult male mice. Because these two pathways are clearly distinct from each other, one may speculate that a combined treatment of selective ER modulators and selective AR modulators might be beneficial in the treatment of osteoporosis.

Sex steroids are important not only for the maintenance of the female skeleton, but also for the male skeleton. The relative contribution of androgens versus estrogens in the regulation of the male skeleton is unclear. Testosterone replacement therapy increases bone mineral density (BMD) in hypogonadal men (1), but several clinical studies indicate that BMD is correlated more to serum levels of estradiol than to serum levels of testosterone in males (2–4). A previous clinical study, which directly compared estrogen versus testosterone effects on bone, showed that estrogens play the dominant role in the regulation of bone resorption markers, whereas both estrogens and testosterone contribute to the maintenance of markers for bone formation (5).

The effects of testosterone can be exerted either directly by means of the androgen receptor (AR) or indirectly by aromatization to estrogens and further by estrogen receptor (ER)α and/or ERβ. All three sex steroid receptors are expressed both in growth-plate cartilage and in bone (6–11). Functional studies using sex steroid receptor-inactivated animal models have demonstrated that ERα but not ERβ is important for the regulation of appendicular longitudinal skeletal growth in male mice (12–14), and a recent report indicates that AR-inactivated male mice have unaffected bone length (15).

Orchidectomy (orx) decreases the amount of trabecular and cortical bone in adult rodents. We and others have shown that the trabecular bone-sparing effect of estrogens is present in orx $ER\beta^{-/-}$ but not in $ER\alpha^{-/-}$ or $ER\alpha^{-/-}\beta^{-/-}$ mice, demonstrating that ERα but not ERβ mediates the trabecular bone-sparing effect of estrogens in male mice (16–18). Interestingly, orx resulted in decreased trabecular volumetric BMD (tvBMD) in $ER\alpha^{-/-}\beta^{-/-}$ mice, indirectly demonstrating that a testicular factor, probably acting by means of the AR, is also of importance for the maintenance of tvBMD in male mice (16).

It was recently demonstrated *in vitro* that the ERs and the AR transmit the antiapoptotic signal in osteoblasts with the same efficiency irrespective of whether the ligand is an estrogen or an androgen (19). To directly compare *in vivo* in males the effect of ER activation on bone with the effect of AR activation, orx WT and ER-inactivated mice were treated with the nonaromatizable androgen 5α-dihydrotestosterone (DHT), 17β-estradiol, or vehicle. We here demonstrate that the *in vivo* effect of ER activation on bone is clearly distinct from the effect of AR activation in adult male mice.

Materials and Methods

Animals. Male and female double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated, resulting in WT, $ER\alpha^{-/-}ER\beta^{+/+}$ ($ER\alpha^{-/-}$), $ER\alpha^{+/+}ER\beta^{-/-}$ ($ER\beta^{-/-}$), and $ER\alpha^{-/-}ER\beta^{-/-}$ ($ER\alpha^{-/-}\beta^{-/-}$) offspring as described (12). 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 feed, and maize germ), vegetable proteins (14.0% hipro soya), and vegetable oil (0.8% soya oil). At 9 months of age, mice were orchidectomized or sham-operated and treated for 4 weeks with 17β-estradiol (0.05 μg/day) or DHT (45 μg/day), administered by means of s.c. silastic implants (Silclear Tubing, Degania Silicone, Jordan Valley, Israel) in the cervical region (20). Vehicle animals received empty implants. 17β-Estradiol and DHT were obtained from Sigma. The Ethics Committee of Göteborg University approved this study.

Peripheral Quantitative Computerized Tomography (pQCT). CT was performed with the pQCT XCT RESEARCH M (Version 4.5B,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ER, estrogen receptor; AR, androgen receptor; BMD, bone mineral density; tvBMD, trabecular volumetric BMD; BMC, bone mineral content; orx, orchidectomy; DHT, 5α-dihydrotestosterone; IGF-I, insulin-like growth factor I; CT, computerized tomography; pQCT, peripheral quantitative computerized tomography.

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Table 1. Weight of seminal vesicles and ventral prostate

Parameter	Sham	orx + V	orx + E	orx + DHT
Seminal vesicle weight, mg				
WT	347.4 ± 26.8	32.6 ± 6.2††	42.7 ± 13.6††	379.2 ± 25.1**
ERα$^{-/-}$β$^{-/-}$	453.8 ± 33.4	134.7 ± 21.2†	211.3 ± 67.6†	615.0 ± 71.8**
Ventral prostate weight, mg				
WT	20.0 ± 2.5	4.0 ± 0.7††	6.2 ± 2.0††	16.6 ± 1.8**
ERα$^{-/-}$β$^{-/-}$	18.7 ± 2.2	6.4 ± 0.3†	6.5 ± 0.4†	28.8 ± 4.3††**

Nine-month-old male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included. n = 7–8 in the WT groups; n = 4–6 in the ERα$^{-/-}$β$^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$ vs. orx + V; ††, $P < 0.01$; †, $P < 0.05$ vs. sham; ANOVA followed by Student–Newman–Keul's multiple-range test.

Norland, Fort Atkinson, WI) operating at a resolution of 70 μm as described (13). Trabecular BMD was determined *ex vivo*, with a metaphyseal pQCT scan of the distal femur. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 4% of the total length of the femur, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were determined *ex vivo* with a middiaphyseal pQCT scan of the femur.

MicroCT. MicroCT analysis was done on the distal femur by using a Skyscan 1072 scanner (Skyscan N.V.) and imaged with an x-ray tube voltage of 50 kV with a 1-mm aluminum filter. The scanning angular rotation was 185° and the angular increment was 0.675°. Pixel size was 4.56 μm and magnification was ×60. Reconstructed datasets were segmented into binary images by using adaptive local thresholding (21). Trabecular bone distal of the growth plate was selected for analysis within a conforming volume of interest (cortical bone excluded), commencing at a distance of 100 μm from the growth plate and extending a further longitudinal distance of 2.3 mm in the proximal direction. The number of slices was 500, each with the same thickness as the pixel size, 4.56 μm. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method (22). Trabecular bone thickness measurement was calibrated by using aluminum foils of 20- and 250-μm thickness (Advent Research Materials, Oxford). For both foils the tolerance of the stated thickness was ±10%. Paired foils of each thickness were scanned, and CT images of them were reconstructed and segmented by using exactly the same steps and parameters as applied to all the mouse femurs in this study. Aluminum provides a suitable material for calibration of microCT measurement of bone-structure thickness. The x-ray opacity of aluminum is slightly greater than that of cortical bone. However, aluminum is materially uniform on a micrometer scale, unlike hydroxyapatite preparations at densities similar to cortical bone, which is important for precise calibration of thicknesses of microns to tens of microns.

Dual X-Ray Absorptiometry. Measurement of areal BMD of the middiaphyseal area of femur *ex vivo* was performed with the Norland pDEXA SABRE (Norland) and the SABRE RESEARCH software (v3.6) as described (13).

Mechanical Testing. The tibia was subjected to mechanical testing by using Mechanical Tester 8841 (Instron, Canton, MA). Three-point bending force was measured by placing the bone horizontally with the anterior surface upward and applying a pressing force vertically to the midshaft of the bone. Each bone was compressed with a constant speed of 2 mm/min until failure. The breaking force (maximal load) was defined as the bending load at failure.

Serum Parameters. Serum osteocalcin levels were measured by using a monoclonal antibody raised against human osteocalcin (Rat-MID osteocalcin ELISA, Osteometer Biotech, Herlev, Denmark). The sensitivity of the osteocalcin assay was 21.1 ng/ml and intra- and interassay coefficients of variation (CVs) were <10%. Serum insulin-like growth factor I (IGF-I) levels were measured by double-antibody, IGF-binding protein-blocked RIA (23).

Statistical Analysis. Data are expressed as mean ± SEM. In general, statistical significance ($P \leq 0.05$) was determined by using one-way ANOVA followed by Student–Newman–Keul's multiple-range test. Two-way ANOVA, in which ERα$^{-/-}$ and ERβ$^{-/-}$ were regarded as separate treatments, followed by Student–Newman–Keul's multiple-range test, was used for the analyses of the endosteal and periosteal circumferences. Linear regression analyses were performed with serum IGF-I as the independent variable and cortical or trabecular bone parameters as the dependant variable. Pearson's correlation coefficient (r) was calculated. Student's t tests were used to analyze data from the linear-regression analyses, and $P < 0.05$ was considered significant.

Results

Effect of Orchidectomy in WT Mice. As expected, the weights of the seminal vesicles and the ventral prostate were reduced by orx in the WT mice (Table 1). Furthermore, orx resulted in a reduction of both tvBMD, as measured by pQCT, and bone volume, as measured by microCT in the metaphyseal region of femur; Figs. 1–3), and cortical [bone mineral content (BMC), cross-sectional area, thickness, and density as measured by a middiaphyseal pQCT scan of the femur; Table 2] bone parameters. These orx-induced structural bone changes were associated with reduced mechanical strength, as measured by using three-point bending of the tibia (Fig. 4) and with increased serum levels of osteocalcin (Fig. 5*A*).

Effect of 17β-Estradiol and DHT in Orchidectomized WT Mice. ***Seminal vesicles and the ventral prostate.*** The DHT dose used (45 μg/day) was physiological because it completely prevented the orx-induced loss of weight of the seminal vesicles and the ventral prostate in WT mice (Table 1). As expected, 17β-estradiol did not prevent the orx-induced weight loss of the seminal vesicles or the ventral prostate (Table 1).

Trabecular bone. The capacity of 17β-estradiol and DHT to preserve the trabecular and cortical bone was then compared. The tvBMD was first measured in the metaphyseal region of the distal femur by using pQCT. Both 17β-estradiol and DHT prevented orx-induced reduction in tvBMD in WT mice (Fig. 1). To investigate whether 17β-estradiol and DHT prevent the orx-induced loss of trabecular bone by similar alterations in the trabecular bone microarchitecture, 3D microCT analyses of the metaphyseal region of distal femur were performed. Treat-

Table 2. Cortical parameters

Parameter	Sham	orx + V	orx + E	orx + DHT
BMC, mg/mm				
WT	1.23 ± 0.03	1.07 ± 0.03††	1.26 ± 0.05**	1.01 ± 0.03††
$ER\alpha^{-/-}$	1.04 ± 0.04	1.00 ± 0.02	0.92 ± 0.04	0.97 ± 0.03
$ER\alpha^{-/-}\beta^{-/-}$	1.03 ± 0.05	0.98 ± 0.08	0.98 ± 0.05	0.99 ± 0.02
Density, mg/mm³				
WT	1.168 ± 0.006	1.128 ± 0.010††	1.184 ± 0.007**	1.111 ± 0.009††
$ER\alpha^{-/-}$	1.135 ± 0.010	1.134 ± 0.006	1.116 ± 0.016	1.134 ± 0.010
$ER\alpha^{-/-}\beta^{-/-}$	1.130 ± 0.011	1.108 ± 0.020	1.100 ± 0.013	1.126 ± 0.009
Cross-sectional area, mm²				
WT	1.06 ± 0.02	0.94 ± 0.02†	1.06 ± 0.04**	0.91 ± 0.02††
$ER\alpha^{-/-}$	0.91 ± 0.03	0.89 ± 0.01	0.82 ± 0.03	0.85 ± 0.02
$ER\alpha^{-/-}\beta^{-/-}$	0.91 ± 0.04	0.88 ± 0.06	0.89 ± 0.03	0.88 ± 0.02
Thickness, μm				
WT	200 ± 4	177 ± 3††	200 ± 4**	171 ± 4††
$ER\alpha^{-/-}$	183 ± 5	183 ± 2	175 ± 5	177 ± 5
$ER\alpha^{-/-}\beta^{-/-}$	188 ± 5	175 ± 10	176 ± 4	183 ± 2

Cortical bone parameters as determined by using a middiaphyseal pQCT scan of the femur. Nine-month-old WT, $ER\alpha^{-/-}$, and $ER\alpha^{-/-}\beta^{-/-}$ male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included. n = 7–8 in the WT and $ER\alpha^{-/-}$ groups; n = 4–6 in the $ER\alpha^{-/-}\beta^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$ vs. orx + V; ††, $P < 0.01$; †, $P < 0.05$ vs. sham; ANOVA followed by Student–Newman–Keul's multiple-range test.

ment with 17β-estradiol prevented orx-induced reduction in the trabecular bone volume as a result of both preserved number and preserved thickness of trabeculae in WT mice (Figs. 2 and 3). DHT treatment also preserved the trabecular bone volume but, in contrast to 17β-estradiol treatment, it was only caused by preserved trabecular number, whereas the trabecular thickness was unaffected by DHT treatment (Figs. 2 and 3). Thus, even though both 17β-estradiol, by activation of ERs, and DHT, by activation of the AR, exert trabecular bone-sparing effects, it is clear that the underlying mechanisms of action differ as the trabecular thickness is increased by 17β-estradiol but not by DHT.

Cortical bone. Cortical bone parameters were first measured by a middiaphyseal pQCT scan of the femur. Cortical BMC was preserved by 17β-estradiol but not by DHT in orx WT mice (Table 2). The stimulatory effect of 17β-estradiol was due both to an increased cortical volumetric BMD and to an increased cortical cross-sectional area, the latter as a result of an effect on cortical thickness in orx mice (Table 2). DHT did not have any effect on cortical bone parameters in these 10-month-old orx mice (Table 2). The effects on cortical bone were also analyzed by a middiaphyseal dual x-ray absorptiometry scan of the femur, confirming the pQCT findings that 17β-estradiol but not DHT preserved the amount of cortical bone in orx WT mice (cortical areal BMD: ovx plus 17β-estradiol, +12.1 ± 2.7% over vehicle, $P < 0.05$; orx plus DHT, +1.1 ± 5.2% over vehicle, not significant; ANOVA, followed by Student–Newman–Keul's multiple-range test).

Mechanical strength. Mechanical strength, as measured by using three-point bending of the tibia and given as maximal load, was preserved by 17β-estradiol, but not by DHT, in orx WT mice (Fig. 4). The qualitative bone parameter elastic modulus was not affected by 17β-estradiol or DHT treatment in either genotype (data not shown).

Serum osteocalcin and IGF-I. DHT, but not 17β-estradiol, prevented the increase in osteocalcin that followed orchidectomy (Fig. 5*A*). 17β-Estradiol increased, whereas DHT slightly decreased, serum levels of IGF-I in orx WT mice (Fig. 5*B*). Furthermore, serum IGF-I levels were positively correlated with all the cortical (BMC, cortical volumetric BMD, cross-sectional area, thickness, maximal load) and trabecular (trabecular thickness) bone parameters that were specifically preserved by 17β-estradiol but not by DHT (Table 3).

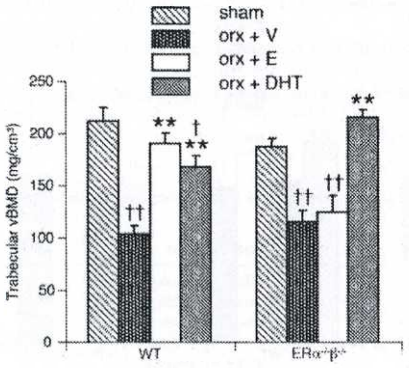

Fig. 1. tvBMD of the distal metaphyseal area of femur measured by using pQCT. Nine-month-old male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included. n = 7–8 in the WT groups; n = 4–6 in the $ER\alpha^{-/-}\beta^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$ vs. orx + V; ††, $P < 0.01$; †, $P < 0.05$ vs. sham; ANOVA followed by Student–Newman–Keuls multiple-range test.

Effect of 17β-Estradiol and DHT in Orchidectomized $ER\alpha^{-/-}\beta^{-/-}$ Mice. Orx resulted in reduced trabecular bone parameters, whereas the cortical bone parameters were mainly unaffected by orx in $ER\alpha^{-/-}\beta^{-/-}$ mice (Figs. 1–3 and Table 2).

Effect of 17β-estradiol. The effect of 17β-estradiol on tvBMD is mediated by ERα as no effect was seen in $ER\alpha^{-/-}\beta^{-/-}$ or $ER\alpha^{-/-}$ mice (Fig. 1, data not shown, and ref. 16). Furthermore, no effect of 17β-estradiol was seen on any other trabecular or cortical bone parameter in orx $ER\alpha^{-/-}\beta^{-/-}$ mice (Figs. 2–4 and Table 2). Statistical analyses performed independently on results from $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice, respectively, showed that the ERα-mediated increase in cortical thickness of femur in orx mice was caused by a decrease in the endosteal circumference (−4.9 ±

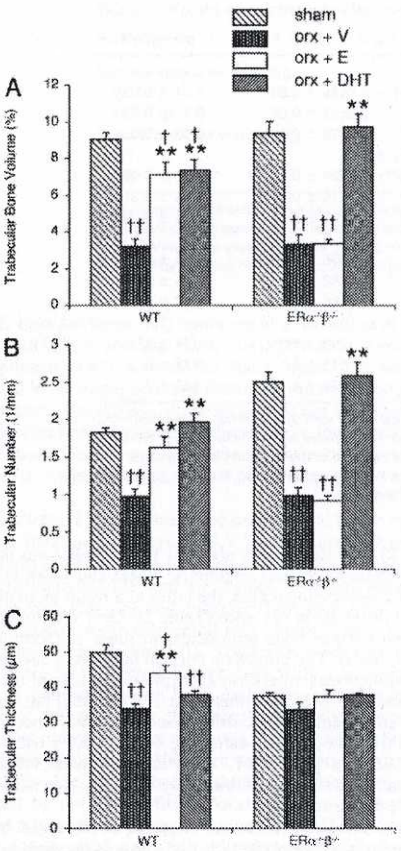

Fig. 2. Trabecular bone volume (*A*), trabecular number (*B*), and trabecular thickness (*C*) as measured by microCT. Nine-month-old male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included. $n = 7–8$ in the WT groups; $n = 4–6$ in the $ER\alpha^{-/-}\beta^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$ vs. orx + V; ††, $P < 0.01$; †, $P < 0.05$ vs. sham; ANOVA followed by Student–Newman–Keul's multiple-range test.

1.6%; $P < 0.05$ two-way ANOVA, followed by Student–Newman–Keul's multiple-range test), whereas the periosteal circumference was not affected. Serum levels of osteocalcin and IGF-I were also not altered by 17β-estradiol in $ER\alpha^{-/-}\beta^{-/-}$ mice (Fig. 5).

Effect of DHT. All the DHT effects seen in orx WT mice were also present in orx $ER\alpha^{-/-}\beta^{-/-}$ mice. Comparison of the effect of DHT in WT versus $ER\alpha^{-/-}\beta^{-/-}$ mice demonstrated that DHT produced a greater effect in $ER\alpha^{-/-}\beta^{-/-}$ mice than in WT mice for the weight of the ventral prostate, the trabecular bone volume, the tvBMD, and the trabecular number ($P < 0.05$, Student's *t* test; Table 1 and Figs. 1 and 2). A similar but not significant tendency was also seen for the weight of seminal vesicles ($P = 0.073$; Table 1).

Discussion

The role of estrogens in the regulation of the adult skeleton in females is well established, whereas the relative importance of

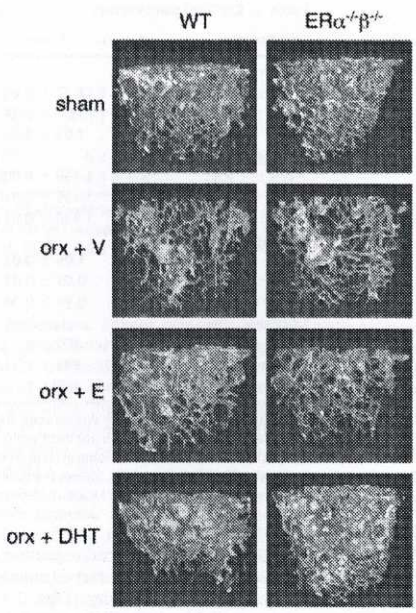

Fig. 3. Representative microCT scans. Nine-month-old male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included.

estrogens versus androgens in the regulation of bone metabolism in adult males remains unclear. We and others have demonstrated that orx-induced trabecular bone loss can be prevented by testosterone, DHT, and 17β-estradiol in adult rodents (16, 17, 20, 24–26). The present study, by using both pQCT and microCT, confirms our previous reports that ERα but not ERβ is of importance for the trabecular bone-sparing effect of estrogens in male mice (16, 17). Furthermore, the trabecular bone-sparing

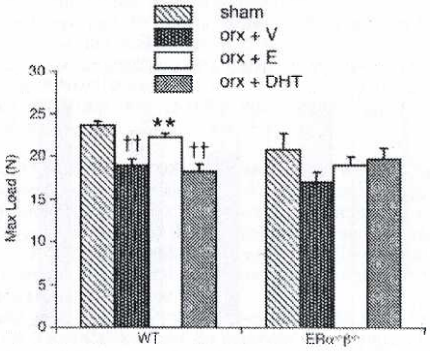

Fig. 4. Maximal load (N), a measurement of the strength of the bone, as measured by using three-point bending of the tibia. Nine-month-old male mice were orchidectomized and then treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were also included. $n = 7–8$ in the WT groups; $n = 4–6$ in the $ER\alpha^{-/-}\beta^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$ vs. orx + V; ††, $P < 0.01$ vs. sham; ANOVA followed by Student–Newman–Keul's multiple-range test.

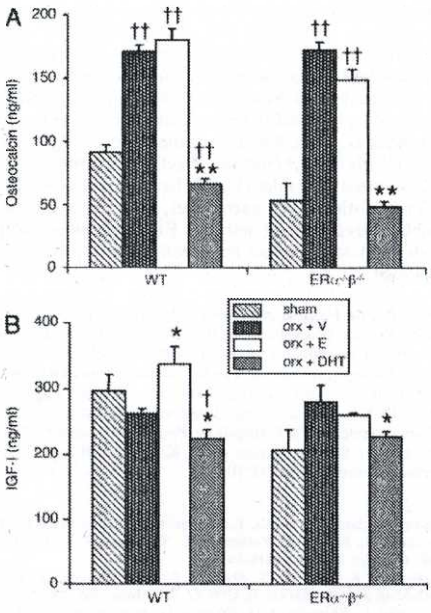

Fig. 5. Serum osteocalcin as measured by ELISA (*A*) and IGF-I as measured by RIA (*B*). Nine-month-old male mice were orchidectomized and treated with vehicle (V), 17β-estradiol (E), or DHT for 4 weeks. Sham-operated animals were included. $n = 7–8$ in the WT groups; $n = 4–6$ in the $ER\alpha^{-/-}\beta^{-/-}$ groups. Values are given as means ± SEM. **, $P < 0.01$; *, $P < 0.05$ vs. orx + V; ††, $P < 0.01$; †, $P < 0.05$ vs. sham; ANOVA followed by Student–Newman–Keul's multiple-range test.

effect of estradiol is only mediated by ERα and not by the AR. In addition, we directly show that the AR-activator DHT increased the amount of trabecular bone independent of ERs. These findings, together with recently published data by Sims *et al.* (18), clearly demonstrate that in contrast to what has been concluded from *in vitro* studies (19, 26), no functional cross-reactivity exists between ERs and AR *in vivo*.

pQCT analyses of tvBMD demonstrated that the trabecular bone-sparing effect of AR activation and ERα activation was of the same magnitude. However, more detailed analysis of the trabecular bone microarchitecture, with high-resolution microCT, demonstrated that ER but not AR activation preserved the trabecular thickness, whereas AR activation only preserved the number of trabeculae (Fig. 6). These findings show that AR activation and ER activation regulate the amount of trabecular bone by different mechanisms of action. This notion is further supported by the different results of 17β-estradiol and DHT treatment for 4 weeks on serum levels of osteocalcin. One possible explanation for this is that the estrogen effect may be mediated not only by decreased bone turnover, but also by increased bone formation, whereas DHT treatment only decreases the bone turnover. In contrast to ovariectomized mice, limited studies have been done on the effect of estradiol on serum levels of osteocalcin and bone turnover in orchidectomized mice. In this regard, we have found that estradiol treatment of orchidectomized mice did not reduce serum levels of osteocalcin (16, 17). Our present findings on serum osteocalcin levels are consistent with our previous data. Moreover, we have found no evidence for reduced bone formation in 17β-estradiol-treated orchidectomized mice as studied by histomorphometric analyses (17). Thus, neither serum levels of osteocalcin nor bone formation, parameters measured by dynamic histomorphometry, were reduced by estradiol treatment in orchidectomized mice. One limitation of the present study is that no marker of bone resorption was measured.

Table 3. Correlation with serum IGF-I and different bone parameters

Compartment	Measurement	*r*
Trabecular	Volumetric BMD	0.06
	Trabecular thickness	0.40**
	Trabecular number	−0.22
Cortical	BMC	0.59***
	Volumetric BMD	0.53***
	Cross-sectional area	0.58***
	Thickness	0.45***
	Maximal load	0.40**
	Elastic modulus	−0.1

Correlations of all animals ($n = 50$) included in the study were calculated by using Pearson's correlation coefficient (*r*). **, $P < 0.01$; ***, $P < 0.001$.

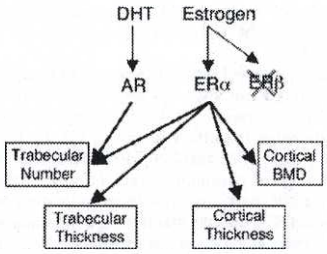

Fig. 6. Differential effects on bone of ERα and AR activation in adult male mice. Activation of ERα preserves the trabecular number, trabecular thickness, cortical thickness, and cortical density, whereas activation of AR preserves only the trabecular number after orx.

An interesting difference between the effect of ER activation and AR activation was also seen on the cortical bone parameters. 17β-Estradiol preserved the amount of cortical bone (cortical cross-sectional area and thickness), the density of the cortical bone, and the mechanical strength of the cortical bone. In contrast, no effect by DHT treatment was seen on any of these cortical bone parameters. The ERα-induced increase in cortical thickness compared with orx mice was caused by a reduced endosteal circumference, whereas the periosteal circumference was unaffected. We and others have recently demonstrated that endocrine, liver-derived IGF-I is an important regulator of the amount of cortical bone and the mechanical strength of cortical bone (27, 28). In the present study, ER activation, but not AR activation, resulted in increased serum levels of IGF-I, which in turn were positively correlated with several cortical bone parameters, suggesting that the effect of 17β-estradiol on these cortical bone parameters might be mediated by an induction of the amount of endocrine IGF-I. Thus, we propose that ER activation, but not AR activation, results in increased serum levels of IGF-I, which in turn increase the amount of cortical bone in orx mice.

The tvBMD and the trabecular bone volume were not correlated with serum IGF-I. However, microCT analyses showed that the thickness of the trabeculae, but not the number of trabeculae, was correlated with serum IGF-I; and because it was the thickness of the trabeculae that was specifically increased by

17β-estradiol, but not by DHT, one may speculate that 17β-estradiol increases the trabecular thickness by an induction of serum IGF-I levels.

The above-mentioned results of sex steroid receptor specificity in bone, derived from the experiment by using replacement therapy with 17β-estradiol or DHT to orx mice, are supported by our observations of the effect of orx in ER double-inactivated mice. Orx of these mice resulted in reduced tvBMD but unaffected cortical bone parameters, indicating that the trabecular bone is, at least in part, maintained by activation of the AR in gonadally intact, ER double-inactivated male mice, whereas AR activation in these mice does not influence cortical bone.

For some androgen-regulated parameters the effect of DHT was greater in $ER\alpha^{-/-}\beta^{-/-}$ mice than in WT mice, which may be because of increased AR expression in $ER\alpha^{-/-}\beta^{-/-}$ mice. AR expression was not analyzed in the present study. Sims *et al.* (18), however, have shown that AR expression was increased in ERα-inactivated mice.

In conclusion, ER activation results in preserved thickness and number of trabeculae and preserved thickness and volumetric density of cortical bone; these effects of 17β-estradiol cannot be mediated by means of the AR (Fig. 6). In contrast, AR activation results in a specific preservation of the number of trabeculae, whereas the thickness of the trabeculae and the cortical bone parameters are unaffected (Fig. 6). 17β-Estradiol increased serum levels of IGF-I, which were positively correlated with all the cortical and trabecular bone parameters that were specifically preserved by 17β-estradiol but not by DHT, suggesting that IGF-I might be involved in the mechanism behind these effects of 17β-estradiol. Thus, the bone-sparing effect of ER activation *in vivo* is clearly distinct from the effect of AR activation in adult gonadectomized male mice (Fig. 6). Because these two pathways are clearly distinct from each other, one may speculate that a combined treatment of selective ER modulators and selective AR modulators might be beneficial in the treatment of osteoporosis.

We thank Anette Hansevi and Maud Petersson for excellent technical assistance. We also thank the SWEGENE Center for Bio-Imaging (CBI) and Göteborg University for technical support regarding image analysis. This study was supported by the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, European Commission Grant QLK4-CT-2002-02528, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation, the Swedish Cancer Fund, Karo Bio AB, and Katholieke Universiteit Leuven Grant OT/01/39.

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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 19, Number 11, 2004
Published online on August 23, 2004; doi: 10.1359/JBMR.040819

Additive Protective Effects of Estrogen and Androgen Treatment on Trabecular Bone in Ovariectomized Rats

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ABSTRACT: Both ER and AR activation regulates trabecular bone mass. We show that combined estrogen and androgen treatment results in additive protection of trabecular bone in OVX rats. This may in part be attributable to the effect of AR activation to attenuate the inhibitory effect of ER activation on bone formation.

Introduction: Sex steroids are important regulators of trabecular bone mass. Both estrogen receptor (ER) and androgen receptor (AR) activation results in increased trabecular bone mass. The aim of this study was to investigate if combined estrogen and androgen treatment might be beneficial in the treatment of trabecular bone loss.
Materials and Methods: Twelve-week-old female rats were ovariectomized (OVX) and treated with vehicle (V), 17β-estradiol (E_2; ER activation), dihydrotestosterone (DHT; AR activation), or the combination (E_2 + DHT) for 6 weeks. The skeletal phenotype was analyzed by pQCT, μCT, histomorphometry of growth plates, and serum levels of biochemical bone markers.
Results: Both E_2 (+121% over V) and DHT (+34%) preserved the trabecular volumetric BMD (tvBMD) in OVX rats. The effect of E_2 and DHT on tvBMD was additive, resulting in a 182% increase over V in the rats given E_2 + DHT. μCT analyses of the trabecular bone microstructure revealed that the effect of E_2 and DHT was additive on the number of trabeculae. E_2 treatment reduced serum markers of both bone resorption (collagen C-terminal telopeptide) and bone formation (osteocalcin), indicating reduced bone turnover. Addition of DHT to E_2 treatment did not modulate the effects of E_2 on the marker of bone resorption, whereas it attenuated the inhibitory effect of E_2 on the bone formation marker, which might explain the additive protective effect of E_2 and DHT on trabecular bone mass. In contrast, DHT partially counteracted the suppressive effect of E_2 on longitudinal bone growth and the E_2-induced alterations in growth plate morphology.
Conclusions: These findings show that combined estrogen and androgen treatment results in additive protective effects on trabecular bone in OVX rats. Our data suggest that a combined treatment with selective ER and AR modulators might be beneficial in the treatment of osteoporosis.
J Bone Miner Res 2004;19:1833–1839. Published online on August 23, 2004; doi: 10.1359/JBMR.040819

Key words: estrogens, androgens, ovariectomy, trabecular bone, longitudinal bone growth

INTRODUCTION

OSTEOPOROSIS IS A COMMON disorder characterized by reduced bone mass and an increased susceptibility to fractures. Traditionally it has been considered a condition of postmenopausal and elderly women, but osteoporosis in men is now recognized as an increasingly important public health issue.[1] Sex steroids are important for the maintenance of both the female and the male skeleton.[1–3] However, the relative contribution of androgens versus estrogens in the regulation of the skeleton is unclear. The effects of testosterone can be exerted either directly through the androgen receptor (AR) or indirectly through aromatization to estrogens and further through estrogen receptor (ER)α and/or ERβ.[4] All three of these sex steroid receptors are expressed both in growth plate cartilage and in bone.[5–9] ERα is the major ER responsible for the trabecular bone–sparing effect of estrogen both in males and females.[10–13] Studies using ER-inactivated mice have shown that ERβ modulates ERα-mediated gene transcription, supporting a "Ying Yang" relationship between ERα and ERβ in the regulation of bone mass in female mice.[14–17] The nonaromatizable androgen dihydrotestosterone (DHT) increases trabecular bone mass in mice devoid of ERs, showing that the AR, besides ERs, has the capacity to preserve trabecular bone in gonadectomized mice.[11,12,18] Some in

The authors have no conflict of interest.

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vitro studies have shown that ERα, ERβ, and AR can transmit the antiapoptotic effect of sex steroids with similar efficiency, irrespective of whether the ligand is an estrogen or an androgen.[19–21] However, several in vivo studies do not support the notion that estrogens have important AR-mediated physiological effects on trabecular bone mass or the concept that non-aromatizable androgens exert bone-sparing effects on trabecular BMD through direct activation of the ERs.[10–12,18,22–24] Furthermore, it was recently shown that the in vivo effect of ER activation on bone is distinct from the effect of AR activation.[11,12,25] Because these two pathways are distinct from each other, one may speculate that a combined treatment with selective ER and AR modulators might be beneficial in the treatment of osteoporosis.[12] In this study, we have examined the effects of combined estrogen and androgen treatment on trabecular bone mass and longitudinal bone growth in ovariectomized (OVX) rats.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were purchased from Scanbur BK AB (Sollentuna, Sweden). The animals were housed in a temperature- and humidity-controlled room with a 6:00 a.m. to 6:00 p.m. light cycle and allowed a soy-free diet containing 0.7% of calcium and 0.5% of phosphorus (R70; Lactamin AB, Kimstad, Sweden) and tap water ad libitum. All procedures were approved by the ethics committee at Göteborg University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Study protocol

The animals were randomly divided into five groups: sham operation + vehicle treatment (sham, $n = 12$), OVX + vehicle treatment (V, $n = 10$), OVX + 17β-estradiol treatment (E_2, $n = 11$), OVX + dihydrotestosterone treatment (DHT, $n = 10$), and OVX + combined E_2 and DHT treatment (E_2 + DHT, $n = 11$). At 12 weeks of age (body weight, 251 ± 2 g), the rats were either sham-operated or OVX under isoflurane anesthesia (Baxter Medical AB, Kista, Sweden), and small silastic implants were placed subcutaneously in the cervical region. The silastic implants were prepared as previously described,[25] releasing 2.5 μg/day of E_2 or 40 μg/day of DHT. Vehicle-treated animals received an empty implant and the E_2 + DHT group received both an E_2 and a DHT implant. 17β-estradiol and 5α-dihydrotestosterone were obtained from Sigma Chemical. After 3 weeks of treatment, blood samples for subsequent serum analyses of collagen C-terminal telopeptide, osteocalcin, and insulin-like growth factor-I (IGF-I) were collected from the tail. After 6 weeks of treatment, the animals were killed by excision of the heart under isoflurane anesthesia, and bones were collected.

pQCT

pQCT was performed with the Stratec pQCT XCT Research M (Norland; v5.4B) operating at a resolution of 70 μm.[26] Trabecular volumetric BMD (tvBMD) was determined ex vivo, with a metaphyseal pQCT scan of the distal femur, and defined as the inner 45% of the total cross-sectional area. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 7% of the total length of the femur. Cortical bone parameters were determined ex vivo with a mid-diaphyseal pQCT scan of the femur. The CVs for these measurements were <2%.

μCT

μCT analysis was done on the distal femur using the Skyscan 1072 scanner (Skyscan N.V., Aartselaar, Belgium), imaged with an X-ray tube voltage of 70 kV and current 100 μA, with a 1-mm aluminum filter. Femurs were taken from alcohol storage and dried in air for 1 h before scanning. The scanning angular rotation was 185°, and the angular increment was 0.45°. The voxel size was 7.66 μm isotropically and the magnification was ×37. Data sets were reconstructed using a modified Feldkamp algorithm[27] and segmented into binary images using adaptive local thresholding.[28] Trabecular bone distal to the growth plate was selected for analysis within a conforming volume of interest (cortical bone excluded) commencing at a distance of 1.5 mm from the growth plate, and extending a further longitudinal distance of 2.5 mm in the proximal direction. A total of 330 image slices was analyzed for each femur. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method.[29] Measurements of trabecular thickness were calibrated by scanning and analyzing three aluminum foils with thicknesses of 50, 125 and 250 μm.

Quantitative histology of growth plates

The length of the femur and tibia was measured ex vivo using vernier calipers (Helios; Promet CEJ AB, Eskilstuna, Sweden). Quantitative histology of the growth plate was analyzed as previously described,[30] with slight modifications. Briefly, the right proximal tibia was fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Sections (5 μm thick) were stained with Alcian blue/van Gieson. Images were captured using a Nikon Eclipse E800 light microscope connected to a Hamamatsu digital camera C4742–95 and a computer. All histological measurements were performed in the central two-thirds of the growth plate sections at ×200 magnification using Olympus MicroImage software (version 4.0; Olympus Optical, Hamburg, Germany). The height of the growth plate was calculated as an average of 25 measurements per growth plate. The number of proliferative and hypertrophic cells was counted in 15 columns per growth plate and averaged for each growth plate. Hypertrophic chondrocytes were defined by a height of >7 μm. The height of the terminal hypertrophic chondrocyte, the cell in the last intact lacuna, was measured in 25 different columns per growth plate and averaged.

Serum parameters

Serum levels of collagen C-terminal telopeptide, as a marker of bone resorption, were measured using an ELISA (RatLaps; Nordic Bioscience Diagnostics, Herlev, Denmark). The intra- and interassay CVs were <11%. Serum

TABLE 1. BODY WEIGHT AND UTERINE WEIGHT

	Sham	*V*	*E_2*	*DHT*	*E_2 + DHT*
Body weight (g)	286 ± 6	330 ± 9*	268 ± 4*†	329 ± 10*‡	277 ± 4†§
Uterine weight (mg)	529 ± 48	145 ± 10*	923 ± 116*†	186 ± 16*‡	867 ± 37*†§

Twelve-week-old female rats were OVX and treated with vehicle (V), 17β-estradiol (E_2), dihydrotestosterone (DHT), or the combination (E_2 + DHT) for 6 weeks. Sham-operated animals were also included (Sham). n = 10–12. Values are given as means ± SE.

* $p < 0.05$ vs. sham.

† $p < 0.05$ vs. V.

‡ $p < 0.05$ vs. E_2.

§ $p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

osteocalcin levels were measured using an IRMA (Immutopics, San Clemente, CA, USA). The intra- and interassay CVs were <5%. Serum was assayed for IGF-I by a double antibody IGF binding protein-blocked RIA (Mediagnost, Tubingen, Germany).

Statistical analysis

A p value of <0.05 was considered statistically significant. All statistical comparisons between the five groups were performed using a one-way ANOVA and posthoc testing with Fisher's protected least significance difference test. A linear regression analysis was performed with serum IGF-I as the independent variable and height of hypertrophic chondrocytes as the dependent variable, and Pearson's correlation coefficient (r) was calculated.

RESULTS

Body weight and uterine weight

As expected, the uterine weight was clearly reduced by OVX. The E_2 dose chosen prevented the loss in uterine weight and even resulted in higher uterine weight in the E_2 group compared with sham rats (Table 1). DHT treatment did not significantly affect the uterine weight. OVX resulted in an increased body weight, which was prevented by E_2 but not by DHT treatment (Table 1).

tvBMD

tvBMD was measured in the metaphyseal region of the distal femur using pQCT. OVX resulted in a pronounced loss of tvBMD (−45%, $p < 0.01$, Fig. 1) compared with sham rats. E_2 treatment of the OVX rats fully prevented the OVX-induced trabecular bone loss (+121% over OVX vehicle) and even resulted in slightly higher tvBMD than observed in sham rats. DHT treatment increased (+34%) the tvBMD compared with vehicle-treated OVX rats, but it only partially prevented the OVX-induced loss of tvBMD. Interestingly, the protective effect of E_2 and DHT was additive, resulting in a 182% increase in tvBMD of the rats given E_2 + DHT compared with OVX (Fig. 1). In contrast, no interaction between E_2 and DHT was seen for the cortical bone parameters as measured by a diaphyseal pQCT section of the femur (data not shown).

Structural parameters of trabecular bone measured by μCT

To investigate the effects of the different treatments on trabecular bone microarchitecture, 3D μCT analyses of the metaphyseal region of the distal femur were performed. Ovariectomy resulted in a pronounced reduction in trabecular bone volume, mainly as a result of a reduced number of trabeculae, whereas no major effect was seen on the thickness of the trabeculae (Figs. 2 and 3). E_2 treatment fully and DHT treatment partially prevented the OVX-induced reduction in trabecular bone volume (Fig. 2A). Both E_2 and DHT increased the number of trabeculae, whereas only E_2 increased the thickness of the trabeculae (Figs. 2B and 2C). The effect of E_2 and DHT on trabecular bone volume was

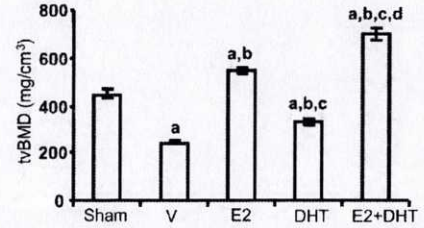

FIG. 1. tvBMD of the distal metaphyseal area of the femur measured using pQCT. Twelve-week-old female rats were OVX and treated with V, E_2, DHT, or E_2 + DHT for 6 weeks. Sham-operated animals were also included (sham). n = 10–12. Values are given as means ± SE. [a]$p < 0.05$ vs. sham; [b]$p < 0.05$ vs. V; [c]$p < 0.05$ vs. E_2; [d]$p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

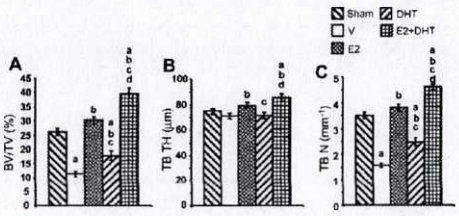

FIG. 2. (A) BV/TV, (B) Tb.Th, and (C) Tb. N as measured by μCT. Twelve-week-old female rats were OVX and treated with V, E_2, DHT, or E_2 + DHT for 6 weeks. Sham-operated animals were also included (sham). n = 10–12. Values are given as means ± SE. [a]$p < 0.05$ vs. sham; [b]$p < 0.05$ vs. V; [c]$p < 0.05$ vs. E_2; [d]$p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

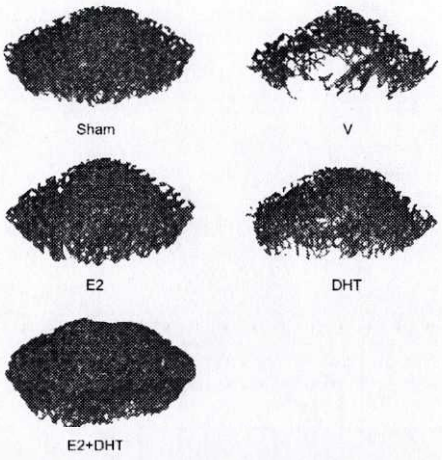

FIG. 3. Representative μCT scans of OVX rats treated with V, E_2, DHT, or E_2 + DHT for 6 weeks. Sham-operated animals were also included (sham).

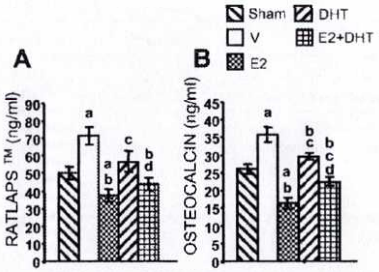

FIG. 4. Serum levels of (A) collagen C-terminal telopeptide (RatLaps) and (B) osteocalcin 3 weeks after OVX. Twelve-week-old female rats were OVX and treated with V, E_2, DHT, or E_2 + DHT. Sham-operated animals were also included (sham). $n = 10–12$. Values are given as means ± SE. $^a p < 0.05$ vs. sham; $^b p < 0.05$ vs. V; $^c p < 0.05$ vs. E_2; $^d p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

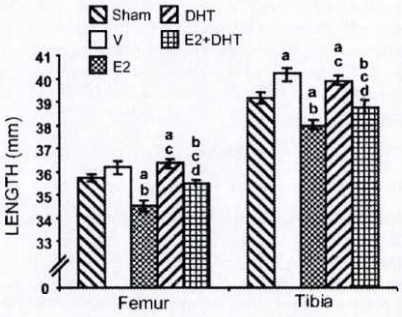

FIG. 5. Length of the femur and tibia. Twelve-week-old female rats were OVX and treated with V, E_2, DHT, or E_2 + DHT for 6 weeks. Sham-operated animals were also included (sham). $n = 10–12$. Values are given as means ± SE. $^a p < 0.05$ vs. sham; $^b p < 0.05$ vs. V; $^c p < 0.05$ vs. E_2; $^d p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

additive and mainly caused by an additive effect on trabecular number as shown by significantly higher values for these parameters in the E_2 + DHT group compared with both the E_2 and the DHT groups (Figs. 2 and 3).

Serum bone markers

To investigate the mechanism behind the additive effect of E_2 and DHT on trabecular bone mass, serum bone markers associated with bone resorption (collagen C-terminal telopeptide, RatLaps) and bone formation (osteocalcin) were measured on serum samples collected 3 weeks after the initiation of treatment. OVX resulted in a significant increase in collagen C-terminal telopeptide levels in serum compared with sham (Fig. 4A; $p < 0.05$). E_2 as well as E_2 + DHT reduced serum levels of collagen C-terminal telopeptide, whereas no significant effect was seen for DHT alone (Fig. 4A). Serum levels of osteocalcin were increased 3 weeks after OVX (Fig. 4B; $p < 0.05$). E_2 treatment resulted in a pronounced reduction of serum osteocalcin, whereas DHT treatment resulted in only a slight reduction in osteocalcin levels compared with vehicle-treated OVX rats. Animals with combined E_2 + DHT treatment had higher levels of osteocalcin than E_2-treated rats (Fig. 4B).

Longitudinal growth and growth plate morphology

OVX resulted in an increased length of the tibia (Fig. 5). Compared with vehicle-treated OVX rats, E_2 treatment retarded the longitudinal growth of both the tibia and femur, whereas no effect was seen for DHT treatment alone. However, the addition of DHT to E_2 partially counteracted the inhibitory effect of E_2 on longitudinal growth of both the tibia and femur of OVX rats (Fig. 5).

In an attempt to understand the mechanisms by which the different treatments affect the longitudinal growth, quantitative morphological studies of the growth plates in the proximal tibia were performed. E_2 reduced the width of the growth plate compared with vehicle-treated OVX rats, and similarly, as seen for the lengths of the long bones, addition of DHT to E_2 partially counteracted the inhibitory effect of E_2 (Table 2). The E_2-induced reduction in growth plate width was associated with reductions in the number of proliferative and hypertrophic chondrocytes, a reduced ratio of proliferative/hypertrophic chondrocytes, and a clear reduction in the height of terminally differentiated hypertrophic chondrocytes. The addition of DHT to E_2 fully counteracted the E_2-induced reduction in the height of hypertrophic chondrocytes and partially counteracted the inhibitory effect on the number of proliferative chondrocytes, whereas it did not affect the number of hypertrophic chondrocytes (Table 2). Disturbances in longitudinal bone

TABLE 2. GROWTH PLATE MEASUREMENTS AND SERUM IGF-I

	Sham	*V*	*E_2*	*DHT*	*E_2 + DHT*
Growth plate width (μm)	159 ± 4	161 ± 4	124 ± 4*†	174 ± 5*†‡	135 ± 3*†‡§
Proliferative chondrocytes (cells per column)	7.6 ± 0.2	7.8 ± 0.3	5.0 ± 0.2*†	8.0 ± 0.4‡	6.0 ± 0.2*†‡§
Hypertrophic chondrocytes (cells per column)	5.1 ± 0.3	5.2 ± 0.1	4.3 ± 0.2*†	5.8 ± 0.2*‡	4.3 ± 0.2*†§
Proliferative/hypertrophic	1.52 ± 0.07	1.50 ± 0.08	1.19 ± 0.06*†	1.38 ± 0.06‡	1.41 ± 0.07‡
HHC (μm)	14.5 ± 0.4	14.6 ± 0.8	9.7 ± 0.4*†	15.4 ± 0.4‡	13.7 ± 0.5‡§
Serum IGF-I (ng/ml)	689 ± 38	784 ± 66	550 ± 28*†	738 ± 56‡	721 ± 30‡

Quantitative histology of the proximal tibial growth plate and serum levels of IGF-I. Twelve-week-old female rats were OVX and treated with vehicle (V), 17β-estradiol (E_2), dihydrotestosterone (DHT), or the combination (E_2 + DHT) for 6 weeks. Sham-operated animals were also included (Sham). Serum levels of IGF-I were analyzed after 3 weeks of treatment. n = 10–12. Values are given as means ± SE.

* $p < 0.05$ vs. sham.

† $p < 0.05$ vs. V.

‡ $p < 0.05$ vs. E_2.

§ $p < 0.05$ vs. DHT (ANOVA followed by Fisher's protected least significance difference test).

HHC = height of hypertrophic chondrocytes.

growth and growth plate morphology are often associated with alterations in the growth hormone/IGF-I axis. E_2 reduced serum levels of IGF-I, and this inhibitory effect on IGF-I was counteracted by the addition of DHT (Table 2). Interestingly, serum levels of IGF-I were strongly correlated to the height of the hypertrophic chondrocytes ($r = 0.42$, $p < 0.001$).

DISCUSSION

Osteoporosis is often associated with trabecular bone loss.[31] Both ER activation and AR activation result in a preserved trabecular bone mass.[11,12,24] This study showed that combined estrogen and androgen treatment results in additive protective effects on trabecular bone in OVX rats, suggesting that a combined targeting of both the ER and the AR pathway might be beneficial in the treatment of trabecular bone loss.

Loss of estrogens or androgens increases the rate of bone remodeling by removing restraining effects on osteoblastogenesis and osteoclastogenesis and also causes a focal imbalance between resorption and formation by prolonging the lifespan of osteoclasts and shortening the lifespan of osteoblasts.[32] Although androgens, through the AR, and estrogens, through the ERs, can exert these effects, their relative contribution remains uncertain. Recent studies suggest that androgen action on trabecular bone depends on (local) aromatization of androgens into estrogens.[4,33] However, at least in rodents, androgen action on cancellous bone can be directly mediated through AR activation, even in the absence of ERs.[11,12] This study confirms previous findings that both ER activation and AR activation have the capacity to preserve trabecular bone mass.[11,12,18,24] We also show that both E_2 and DHT prevent the loss of trabeculae, whereas only E_2 protects the thickness of the trabeculae, confirming a previous study using orchidectomized mice.[12]

In contrast to the male skeleton, the effect of androgens on the female skeletal homeostasis has not been well established. However, hyperandrogenic women have increased trabecular BMD, suggesting that androgens also are of importance for the female skeleton.[34] In this study, we showed that DHT treatment results in increased tvBMD in OVX rats, supporting the notion that AR activation is of importance for the regulation of not only the male but also the female skeleton.

Interestingly, we show that combined estrogen and androgen treatment results in an additive protective effect on both the trabecular volumetric BMD, as measured by pQCT, and the trabecular bone volume, as measured by μCT, in females. The additive effect of combined E_2 and DHT treatment on the trabecular bone volume was caused by an increase in the number of trabeculae.

The additive effect on trabecular bone mass and trabecular number might partially be explained by the fact that DHT attenuated the inhibitory effect of E_2 on bone formation, as indicated by the higher levels of osteocalcin in animals with combined E_2 and DHT treatment compared with E_2-treated rats, whereas it did not significantly influence the inhibitory effect of E_2 on bone resorption markers. This would then result in an imbalance with bone formation exceeding bone resorption, resulting in increased bone mass. In line with these data, short-term administration of combined androgen and estrogen treatment reversed the inhibitory effects of estrogen on bone formation markers in postmenopausal women, whereas serum markers of bone resorption were unchanged.[35] Our experimental results, showing additive effects on trabecular bone and similar alterations of serum bone markers as seen in the clinical study, may suggest that combined ER and AR activation could result in an additive effect on trabecular bone mass also in the clinical setting.

The dose of DHT used in this study was selected according to our previous dose-response studies in orchidectomized male rats, and we chose a dose that resulted in prostate weight and seminal vesicle weight of the same magnitude as seen in sham-operated rats.[25] The selected dose of E_2 reversed the OVX-induced loss in uterine weight and even resulted in a higher uterus weight in E_2-treated

compared with sham rats. Thus, the additive effects on trabecular bone seen in this study were achieved by treatment with slightly supraphysiological doses of both E_2 and DHT. One cannot exclude that an effect on trabecular bone of similar magnitude could be reached by monotherapy using even higher doses of E_2 or DHT. However, treatment with higher doses than used in this study would probably be associated with an increased risk of side effects. Therefore, we propose that a combined E_2 and DHT treatment might, in a more specific manner than monotherapy, achieve a target trabecular bone mass without the risk of dose-dependent side effects.

In contrast to the effects on trabecular bone seen in this study, no interactive effects of combined E_2 and DHT treatment were seen on cortical bone parameters. Furthermore, regarding the longitudinal bone growth, DHT counteracted the inhibitory effect of E_2 on longitudinal bone growth in OVX rats. Longitudinal bone growth is dependent on the number of cell divisions of growth plate chondrocytes within each column and the height of the terminally differentiated hypertrophic chondrocytes. Addition of DHT to E_2 fully counteracted the E_2-induced reduction in the height of hypertrophic chondrocytes and partially counteracted the inhibitory effect on the number of proliferative chondrocytes. Interestingly, the inhibitory effect of E_2 and the counteracting effect of addition of DHT to E_2 on growth plate morphology were associated with similar alterations in serum levels of IGF-I, suggesting that the growth hormone/IGF-I axis might be involved in the effects on growth plate morphology. Such a link is further supported by a previous report showing decreased height of hypertrophic chondrocytes in IGF-I knockout mice (i.e., a similar effect as observed in our OVX animals).[36] In accordance with our results on bone length, Coxam et al.[37] found that DHT counteracted the inhibitory effect of E_2 on longitudinal bone growth. In contrast to our results, they found no effect of E_2, DHT, or the combined treatment on trabecular bone volume as assessed by histomorphometry, and thus a possible additive effect of the combined treatment on trabecular bone could not be evaluated. Coxam et al. used a comparable treatment length (8 weeks) and dose of E_2, whereas the doses of DHT were approximately double and 8-fold higher. However, an important difference between our studies is that the treatment was initiated directly after OVX in this study, whereas it was 8 weeks after OVX in the study by Coxam et al.

Our results show that combined estrogen and androgen treatment might result in an additive effect (trabecular bone), no interaction (cortical bone), or an antagonistic effect (longitudinal bone growth) depending on the bone parameter investigated.

In conclusion, combined estrogen and androgen treatment exerts an additive protective effect on trabecular bone in OVX rats. This additive effect on trabecular bone mass might be partially explained by AR activation attenuating the inhibitory effect of ER activation on bone formation. Our findings suggest that a combined treatment of selective ER modulators and selective AR modulators might be beneficial in the treatment of osteoporosis.

ACKNOWLEDGMENTS

This study was supported by the Swedish Research Council (4250, 12206), the Swedish Foundation for Strategic Research, the European Commission Grant QLK4-CT-2002-0258, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, the Novo Nordisk Foundation, the Petrus and Augusta Hedlund's Foundation, the Västra Götaland Foundation, the Göteborg Medical Society, the Tore Nilson Foundation, and Grant OT/01/39 from Research Fund K.U. Leuven. The authors thank Britt-Mari Larsson, Maud Petersson, and Anette Hansevi for excellent technical support.

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Received in original form May 5, 2004; in revised form June 9, 2004; accepted July 2, 2004.

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0888-8809/03/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 17(2):203–208

doi: 10.1210/me.2002-0206

Estrogen Receptor (ER)-β Reduces ERα-Regulated Gene Transcription, Supporting a "Ying Yang" Relationship between ERα and ERβ in Mice

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Estrogen is of importance for the regulation of adult bone metabolism. The aim of the present study was to determine the role of estrogen receptor-β (ERβ) *in vivo* on global estrogen-regulated transcriptional activity in bone. The effect of estrogen in bone of ovariectomized mice was determined using microarray analysis including 9400 genes. Most of the genes (95% = 240 genes) that were increased by estrogen in wild-type (WT) mice were also increased by estrogen in ERβ-inactivated mice. Interestingly, the average stimulatory effect of estrogen on the mRNA levels of these genes was 85% higher in ERβ-inactivated than in WT mice, demonstrating that ERβ reduces estrogen receptor-α (ERα)-regulated gene transcription in bone. The average stimulatory effect of estrogen on estrogen-regulated bone genes in ERα-inactivated mice was intermediate between that seen in WT and ER$\alpha\beta$ double-inactivated mice. Thus, ERβ inhibits ERα-mediated gene transcription in the presence of ERα, whereas, in the absence of ERα, it can partially replace ERα. In conclusion, our *in vivo* data indicate that an important physiological role of ERβ is to modulate ERα-mediated gene transcription supporting a "Ying Yang" relationship between ERα and ERβ in mice. (*Molecular Endocrinology* 17: 203–208, 2003)

ESTROGEN IS OF importance for the regulation of adult bone metabolism. We and others have previously demonstrated that female estrogen receptor-β (ERβ)-inactivated or ER$\alpha^{+/+}\beta^{-/-}$ mice (BERKO) have an increased amount of cortical bone and are partly protected against age-related trabecular bone loss that normally occurs in old female wild-type (WT) mice. Therefore, one may speculate that ERβ represses the bone-protective effect of estrogen mediated via estrogen receptor-α (ERα) (1–3). A possible interaction between ERα and ERβ in the regulation of transcriptional activity has previously been investigated in different *in vitro* systems. It is clear that ERβ and ERα can form heterodimeric complexes with retained DNA-binding ability (4). Transient transfection assays have demonstrated that ERβ has the capacity to repress the transcriptional activity of ERα (5, 6). Furthermore, when complexed with estradiol, ERα and ERβ signal in opposite directions from an activator protein 1 site (7). An inhibitory effect of ERβ might be explained by the finding that ERβ does not contain a strong activation function 1 within its amino terminus but, rather, contains a repressor domain; when this particular segment is removed, the overall transcriptional activity of the receptor increases (6). Thus, previous *in vitro* data indicate that ERβ can sometimes act as a dominant negative regulator of ERα activity. Gene expression profiling opens up possibilities to investigate the interplay between ERα and ERβ in a global sense. The aim of the present study was to determine, *in vivo*, the role of ERβ and its possible interactions with ERα in global estrogen-regulated transcriptional activity in bone.

Abbreviations: BERKO, ER$\alpha^{+/+}\beta^{-/-}$; DERKO, ER$\alpha^{-/-}\beta^{-/-}$; ER, estrogen receptor; ERKO, ER$\alpha^{-/-}\beta^{+/+}$; EST, expressed sequence tag; GR, glucocorticoid receptor; ovx, ovariectomized; PR, progesterone receptor; WT, wild-type.

RESULTS AND DISCUSSION

ERβ Reduces ERα-Regulated Gene Transcription in Bone and Liver

The effects of estrogen on global gene expression in humerus from ovariectomized (ovx) mice were determined by microarray analysis. Most of the genes (95%, 240 genes) that were increased by estrogen in WT mice were also increased by estrogen in BERKO mice, indicating that ERβ, in the presence of ERα, is generally not required for estrogen-regulated gene transcription in bone. The relative magnitude of the effect of estrogen in WT and BERKO mice was compared for these genes. Interestingly, the average stimulatory effect of estrogen on the expression of these genes was 85% higher in BERKO than in WT mice and, when considering the individual genes, it was found that 80% of the genes were more regulated by

estrogen in BERKO than in WT mice (Fig. 1A and Table 1). ERα mRNA levels in bone were unchanged in 3-month-old estrogen-treated female BERKO mice compared with WT mice (WT, 100 ± 13%, BERKO, 132 ± 29% of WT mice, n = 7–8) as determined by real-time PCR.

To study whether the effect of ERβ to reduce estrogen-stimulated gene transcription was specific for bone or whether it could be found in other tissues as well, the effect of estrogen on estrogen-regulated genes was analyzed in the liver. In accordance with the results from the humerus, the average stimulatory effect of estrogen in liver was increased in BERKO compared with WT mice (184% of WT) and when the individual genes were considered it was found that 76% of the genes were more regulated by estrogen in BERKO than in WT mice (Fig. 1B and Table 1). As a negative control, we compared the expression of all genes not regulated by estrogen (n = 9060) without finding any difference between WT and BERKO mice (data not shown). The stimulatory effect of estrogen on the expression of 13 genes in bone and 8 genes in liver was confirmed by real-time PCR analysis (Fig. 2 and data not shown). Thus, in the presence of ERα, it is clear that ERβ reduces the global estrogen-stimulated gene transcription in bone and liver. This is the first demonstration that ERβ is a global inhibitor of ERα-regulated gene transcription in mice. In contrast, a minority of the estrogen-regulated genes were more regulated in WT than in BERKO mice, which might indicate that ERβ has an additional role as a potent activator of a small specific gene set. However, we believe, rather, that these results represent a random distribution of several estrogen-regulated genes, where most genes are more regulated and very few are less regulated in BERKO than in WT mice.

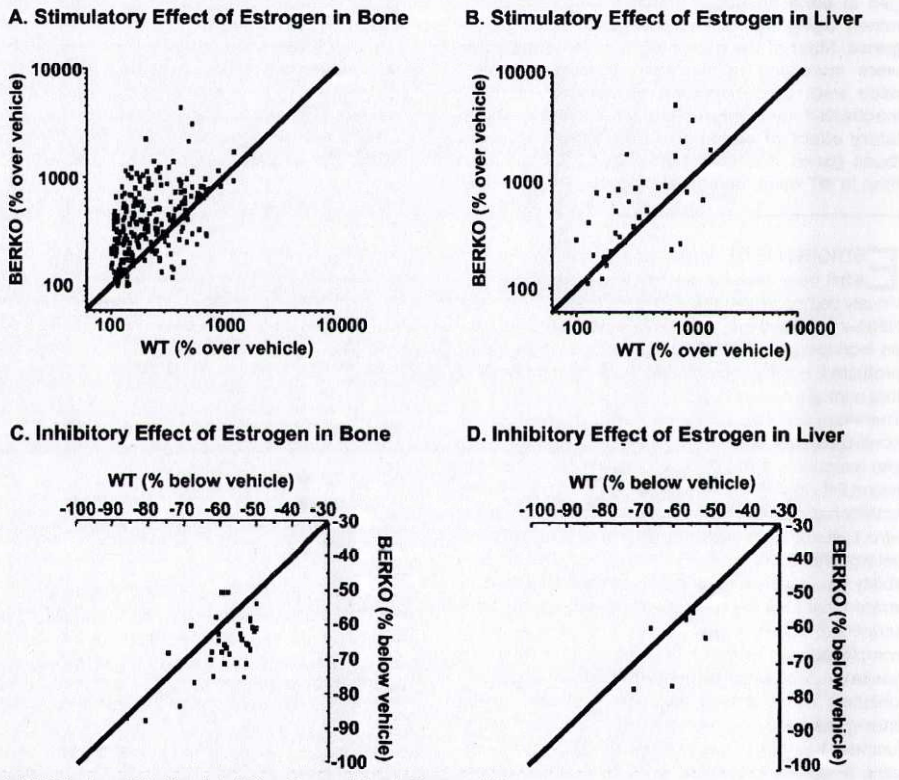

Fig. 1. ERβ Reduces ERα-Regulated Gene Transcription in Bone and Liver

Stimulatory (A and B) and inhibitory (C and D) effects of estrogen on estrogen-regulated gene transcription in bone (A and C) and liver (B and D) of ovx WT and BERKO mice as measured using microarray analysis. The percent increase over vehicle (A and B) or decrease below vehicle (C and D) for each individual estrogen-regulated gene is given for WT (x-axis) and BERKO (y-axis) mice. The *black line* indicates the theoretical position of genes regulated by estrogen to the same magnitude in WT and BERKO mice.

A global ERα-repressing effect of ERβ on transcriptional activity is supported by recent functional *in vivo* studies demonstrating that ERβ represses some ERα-mediated effects, including effects on female bone, thymus involution, fat reduction, and proliferative effects in uterus and prostate (8, 9).

The number of genes inhibited by estrogen in bone from both WT and BERKO mice was lower than the number of genes stimulated by estrogen (stimulated, 240; inhibited, 35). The average inhibitory effect of estrogen on these genes was more pronounced in BERKO compared with WT mice and, when considering the individual genes, it was found that 83% of the genes were more regulated by estrogen in BERKO than in WT mice (Fig. 1C and Table 1). The number of genes inhibited by estrogen in liver (n = 6) was too low to permit any statistical difference between WT and BERKO mice even though a similar tendency as in bone was found (Fig. 1D and Table 1). These data indicate that ERβ, in the presence of ERα, not only reduces the stimulatory but also the inhibitory effect of estrogen on gene transcription.

Table 1. Effect of Estrogen in WT and BERKO Mice

	WT (%)	BERKO (%)
Stimulation in bone (n = 240)	275 ± 14	511 ± 30[a]
Stimulation in liver (n = 34)	472 ± 67	868 ± 179[b]
Inhibition in bone (n = 35)	−59 ± 1	−66 ± 2[a]
Inhibition in liver (n = 6)	−60 ± 3	−65 ± 41

Effect of estrogen on estrogen-regulated gene transcription in bone and liver of ovx mice as studied by microarray analysis. Values are the average effect of estrogen expressed as percent stimulation above or inhibition below vehicle and are given as mean ± SEM. n = number of genes regulated by estrogen in both WT and BERKO mice.

[a] $P < 0.001$ BERKO *vs.* WT, Student's paired *t* test.

[b] $P < 0.05$ BERKO *vs.* WT, Student's paired *t* test.

ERβ Can Partially Replace ERα in Estrogen-Regulated Gene Transcription

The function of ERβ on estrogen-regulated gene transcription in the absence of ERα was investigated by comparing the effect of estrogen in bone from ovx WT, $ER\alpha^{-/-}\beta^{+/+}$ (ERKO), BERKO, and $ER\alpha^{-/-}\beta^{-/-}$ (DERKO) mice. We could, by microarray analysis, clearly demonstrate that more than 98% of the genes stimulated by estrogen in WT and BERKO mice were unchanged by estrogen in DERKO mice, demonstrating that, for most genes, either ERα or ERβ is required for the mediation of the effect of estrogen (Fig. 3). Less than 2% of the estrogen-regulated genes were regulated by estrogen also in DERKO mice, indicating that these few genes were regulated in an ERα/ERβ-

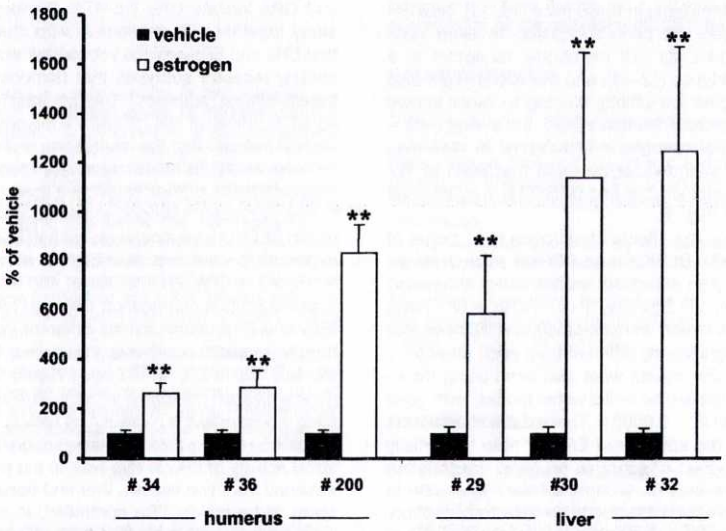

Fig. 2. TaqMan Verification of DNA Microarray Analysis

Verification of DNA microarray analysis with relation to the stimulatory effect of estrogen on three different genes in bone (gene 34, 36, and 200 according to the supplemental data file) and three other genes in liver (gene 29, 30, and 32 according to the supplemental data file) as measured by real-time PCR on individual mice. Values are shown as percent of vehicle-treated WT ± SEM. Student's *t* test. **, $P < 0.01$, estrogen *vs.* vehicle.

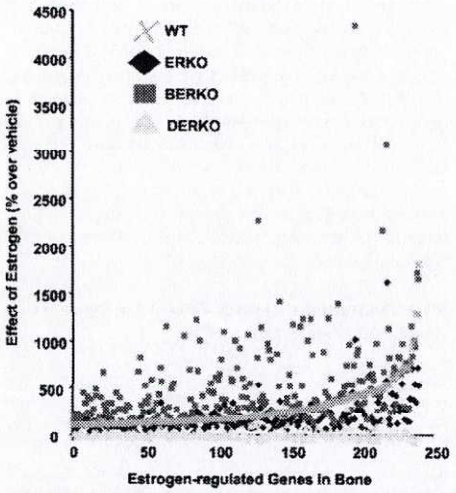

Fig. 3. ERβ Can Partially Replace ERα in Estrogen-Regulated Gene Transcription

Stimulatory effects of estrogen on estrogen-regulated gene transcription in bone of ovx WT, ERKO, BERKO, and DERKO mice as measured using microarray analysis. The percent increase over vehicle for each individual estrogen-regulated gene (n = 240) is given.

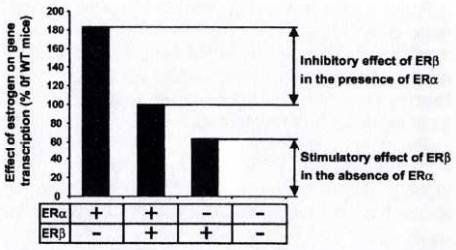

Fig. 4. ERβ inhibits ERα-Mediated Gene Transcription in the Presence of ERα, Whereas It Can Partially Replace ERα in the Absence of ERα

The figure is based on the effects of estrogen in bone of ovx WT, ERKO, BERKO, and DERKO mice. These effects were investigated by a statistical model using three-way ANOVA (gene, n = 240; ERα + or −, ERβ + or −) with gene as block effect, demonstrating that all four groups are significantly different from each other ($P < 0.00001$). The average stimulatory effect of estrogen on the 240 estrogen-regulated genes is given and expressed as percent of WT.

independent manner, but one cannot exclude a minor ERα activity remaining in these mice (10, 11). Alternatively, estrogen has been suggested to exert nongenomic actions via cell membrane receptors in a variety of cell types (12–14), and the effect might also be due to some low-affinity binding to other known nuclear receptors (14). Interestingly, the average stimulatory effect of estrogen in ERKO (176 ± 11% over vehicle) was intermediate between that seen in WT (275 ± 14% over vehicle) and DERKO (−7 ± 3% over vehicle) mice (Fig. 3).

Furthermore, the effects of estrogen in humerus of ovx WT, ERKO, BERKO, and DERKO mice were investigated by a statistical model using three-way ANOVA (gene, n = 240; ERα + or −, ERβ + or −) with gene as block effect, demonstrating that all these four groups are significantly different from each other ($P < 0.00001$). Similar results were also seen using three-way ANOVA applied on ranks within blocks, with gene as block effect ($P < 0.00001$). These data demonstrate that ERβ, in the absence of ERα, is able to partially mediate the effect of estrogen on gene transcription (Fig. 3). However, ERα is more efficient than ERβ in mediating the effect of estrogen on gene transcription. These analyses also demonstrated that there is a clear negative interaction between the effect of ERα and ERβ, which could be interpreted as meaning that ERβ significantly reduces the stimulatory effect of ERα (Figs. 3 and 4). Thus, ERβ inhibits ERα-mediated gene transcription in the presence of ERα, whereas it can partially replace ERα in the absence of ERα (Fig. 4).

It has previously been shown, both for the two progesterone receptor (PR) subtypes (PR-A and PR-B), and for the two glucocorticoid receptor (GR) subtypes (GRα and GRβ), that one of these two subtypes can act as a dominant negative inhibitor of the transcriptional activity of the other subtype [PR-A inhibits PR-B and GRβ inhibits GRα (15–17)]. Our present *in vivo* study together with previous *in vitro* studies suggest that ERα and ERβ provide yet another example of two nuclear receptor subtypes that demonstrate distinct transcriptional activities. The contrasting transcriptional activities of the different subtypes of PR, GR, and ER indicate that the relative tissue distributions of receptor subtypes are a major determinant of the biological effects of their respective ligands.

The increased estrogen-regulated gene transcription in BERKO mice compared with WT mice might be explained by the fact that ERβ is a transdominant repressor of ERα. An alternative, and more probable, explanation is a competition between two activators, ERα and ERβ, which exhibit different intrinsic activities. A competition between the more active ERα and the less active ERβ in WT mice results in a reduced gene activation compared with the situation in BERKO mice where only the more active ERα is present. The interesting finding that ERβ can reduce the transcriptional activity of ERα *in vivo* was, in the present study, obtained from two tissues, liver and bone, which both seem to be mainly ERα controlled. In other tissues, including prostate, lung, and ovary, it is clear that ERβ is a major regulator in its own right (8, 9, 18). Accordingly, we cannot extrapolate the present data to conclude, in general, that repression of ERα is the major physiological function of ERβ in all tissues.

In the present study, mice were treated for 3 wk with estradiol. Therefore, it is not clear which genes were directly transcriptionally regulated by estradiol and which genes were secondarily regulated by other genes regulated by estradiol. Furthermore, the microarray data do not distinguish between changes in transcription *vs.* changes in RNA processing or RNA stability. Thus, the present result should be interpreted as the net result of estrogen on mRNA levels after chronic estradiol treatment.

In conclusion, our data indicate that an important role of ERβ is to modulate ERα-mediated gene transcription in bone and liver, supporting a "Ying Yang" relationship between ERα and ERβ in mice.

MATERIALS AND METHODS

Animals

ER-inactivated mice were bred as previously described (19). Male double-heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated with female double-heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice, resulting in $ER\alpha^{+/+}\beta^{+/+}$ (WT), $ER\alpha^{-/-}\beta^{+/+}$ (ERKO), $ER\alpha^{+/+}\beta^{-/-}$ (BERKO), and $ER\alpha^{-/-}\beta^{-/-}$ (DERKO) offspring (20–22). The diet, housing, and genetic background were as previously described (22). The mice were ovx at 2 months of age. Ovaries were removed after a flank incision and the incisions were closed with metallic clips. After recovery for 4 d, mice were injected sc with 2.3 μg/mouse/d of 17β-estradiol benzoate (Sigma, St. Louis, MO) for 5 d/wk during a 3-wk period. Control mice received injections of vehicle oil (olive oil, Apoteksbolaget, Gothenburg, Sweden). The study protocol was reviewed and approved by the ethical committee at the University of Gothenburg.

DNA Microarray Analysis

RNA from humerus (n = 6: WT vehicle, DERKO estrogen; n = 7: WT estrogen; n = 8: BERKO vehicle, BERKO estrogen; n = 4: DERKO vehicle; n = 3: ERKO vehicle, ERKO estrogen) and liver (n = 6: WT vehicle, WT estrogen, BERKO vehicle, BERKO estrogen, DERKO vehicle and DERKO estrogen; n = 4: ERKO vehicle, ERKO estrogen) were prepared as described elsewhere (23). The RNA was further purified using RNeasy Kit (QIAGEN, Chatsworth, CA). For microarray assays the RNA samples were divided into two pools per animal group, whereas for the confirmatory real-time PCR analysis each individual animal was analyzed separately. The pooled RNA was reverse transcribed into cDNA, labeled, and analyzed by DNA microarray (MG-U74A Array; Affymetrix, Santa Clara, CA). The array represents approximately 5700 characterized mouse genes and approximately 3700 uncharacterized expressed sequence tags (ESTs) (in total, 9400). In the present article we do not distinguish between characterized mouse genes and uncharacterized ESTs. Preparation of labeled cRNA and hybridization was done according to the Affymetrix Gene Chip Expression Analysis manual.

Bioinformatics

Scanned output files were analyzed using Affymetrix Micro Array Suite Version 4.0.1 software. To allow comparison of gene expression, both between groups and between tissues, the GeneChips were globally scaled to an average intensity of 500. The estrogen-regulated genes were determined by calculating the fold change in average between vehicle-treated and estrogen-treated samples. Comparisons were made between the two vehicle-treated and the two estrogen-treated GeneChips, generating a total of four comparisons. We defined very strict criteria for genes to be regarded as regulated; 1) the absolute call for the gene had to be present (*i.e.* regarded by the software to be expressed at a detectable level; Affymetrix Micro Array Suite Version 4.0.1) for all GeneChips; 2) at least three of the four comparisons had to be considered increased (I) or decreased (D) according to Affymetrix algorithms; 3) the average fold increase or decrease of the four comparisons should be at least 2.0-fold. Thus, we may have excluded some estrogen-regulated genes but this was done to avoid false positives among the regulated genes. The average coefficient of variation for the four microarray comparisons was 22.8% for the 240 estrogen-regulated genes in bone.

For determination of the ER specificity of the genes regulated by estrogen in WT mice, the same rules as above were applied for comparisons between vehicle and estrogen-treated ERKO, BERKO, and DERKO GeneChips.

Real-Time PCR Analysis

The confirmatory real-time PCR analyses were, as described in the microarray section, run on each individual sample. The analyses were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden) using probes labeled with the reporter fluorescent dye FAM. Predesigned primers and a probe labeled with the reporter fluorescent dye VIC, specific for 18S rRNA, were included in the reactions as an internal standard. The oligonucleotide primers and probes were purchased from PE Applied Biosystems. The cDNA was amplified at the following conditions: 1 cycle at 50 C for 2 min and 95 C for 10 min, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min. The mRNA amount of each gene was calculated using the Standard Curve Method (multiplex reaction, following the instructions in User Bulletin no. 2, PE Applied Biosystems) and adjusted for the expression of 18S rRNA.

Acknowledgments

Received June 5, 2002. Accepted October 30, 2002.

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This work was supported by the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, the Emil and Vera Cornell Foundation, Petrus and Augusta Hedlunds Foundation, the Swedish Cancer Fund, and Karo Bio AB.

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0026-895X/03/6406-1428–1433$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 64:1428–1433, 2003

Vol. 64, No. 6
2755/1211433
Printed in U.S.A.

Estren Is a Selective Estrogen Receptor Modulator with Transcriptional Activity

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Received June 20, 2003; accepted September 4, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

It was recently reported that the synthetic compound estren increases bone mass without affecting reproductive organs or classic transcription. The aim of the present study was to further characterize the in vivo and in vitro effects of estren. We demonstrate that estren is a selective estrogen receptor modulator (SERM) with a strong effect on thymus, a moderate effect on uterus and trabecular bone, but no major effect on fat or cortical bone in 11-month-old ovariectomized mice. The effect of estren on trabecular bone and uterus is mediated via estrogen receptors (ERs) because no effect is seen in ER double-inactivated mice. Furthermore, with the use of ERα- and ERβ-expressing reporter cell lines, we demonstrate that estren displays an agonistic effect on transcriptional activity of an estrogen-responsive element-driven reporter gene with a degree of agonism similar to that of 17β-estradiol for both ERα and ERβ. Thus, estren has the capacity to exert genomic effects via both ERα and ERβ. We conclude, in contrast to what was previously reported by others, that estren is a SERM with transcriptional activity.

Estrogens and androgens are of importance for the regulation of bone metabolism in both males and females. Skeletal effects of sex steroids are mediated via estrogen receptors (ERs) and the androgen receptor (AR), which are nuclear receptors with transcriptional activity. However, a variety of cell types respond rapidly to estrogens within seconds or minutes, making a classic genomic mechanism of action unlikely (Wehling, 1997; Brubaker and Gay, 1999; McEwen and Alves, 1999; Compston, 2001), and there are now several pieces of evidence supporting the notion of nongenomic functions of sex-steroid receptors (Endoh et al., 1997; Brubaker and Gay, 1999; Migliaccio et al., 2000; Simoncini et al., 2000; Compston, 2001; Duan et al., 2001; Kousteni et al., 2001). Furthermore, Kousteni et al. (2001) proposed that ERα, ERβ, or AR could transmit nongenomic antiapoptotic effects on osteoblasts in vitro with similar efficiency, irrespective of whether the ligand is an estrogen or an androgen. It has been suggested that the genomic mechanisms mediate the reproductive effects, whereas the nongenomic effects are responsible for the bone-sparing effect of estrogens (Kousteni et al., 2001, 2002). The synthetic compound 4-estren-3α,17β-diol (estren) has recently been described to increase bone mass without affecting reproductive organs or classic transcription (Kousteni et al., 2001, 2002). The statement of an absence of transcriptional activity for estren is derived from an in vitro experiment in which estren did not induce C3 transcription in ERα-transfected HeLa cells (Kousteni et al., 2001, 2002). The aims of the present study were the following: 1) to determine the tissue specificity for the effect of estren; 2) to determine whether the in vivo effect of estren is mediated via ERs and/or AR with the same efficiency; and 3) to determine in vitro, using ER-expressing reporter cell lines, whether estren has any transcriptional activity. We demonstrate here that estren is a selective estrogen receptor modulator (SERM) with transcriptional activity.

This study was supported by the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, the European Commission grant QLK4-CT-2002-02528, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, the Emil and Vera Cornell Foundation, Petrus and Augusta Hedlunds Foundation, the Swedish Cancer Fund, and Karo Bio AB.

Materials and Methods

Animals. Male and female double-heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated, resulting in WT, $ER\alpha^{-/-}ER\beta^{+/+}$ ($ER\alpha^{-/-}$), $ER\alpha^{+/+}ER\beta^{-/-}$ ($ER\beta^{-/-}$) and $ER\alpha^{-/-}ER\beta^{-/-}$ ($ER\alpha^{-/-}\beta^{-/-}$) offspring with a mixed C57BL/6J/129 background (Lubahn et al., 1993; Krege et al.,

ABBREVIATIONS: ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; SERM, selective estrogen receptor modulator; AR, androgen receptor; BMD, bone mineral density; BMC, bone mineral content; ovx, ovariectomized; WT, wild type; pQCT, peripheral quantitative computerized tomography; FCS, fetal calf serum; ALP, alkaline phosphatase; 293/hERα, human 293 kidney cells expressing estrogen receptor α; 293/hERβ, human 293 kidney cells expressing estrogen receptor β; ICI 182,780, fulvestrant.

1998). Genotyping of tail DNA was performed at 3 weeks of age as described previously (Vidal et al., 2000). Animals had free access to fresh water and soy-free food pellets (R70; Lactamin AB, Stockholm, Sweden). At 11 months of age, mice were ovariectomized (ovx) and injected daily subcutaneously with 17β-estradiol benzoate (0.7 μg/mouse) (Sigma Chemical, St. Louis, MO) or estren (75 μg/mouse) (Steraloids, Newport, RI) for 4 weeks. Control mice received injections of vehicle oil (olive oil; Apoteksbolaget, Göteborg, Sweden).

Peripheral Quantitative Computerized Tomography. Computerized tomography was performed with the Stratec peripheral quantitative computerized tomography (pQCT) XCT Research M (version 5.4B; Norland Corporation, Fort Atkinson, WI) as described previously (Windahl et al., 1999). Trabecular bone mineral density (BMD) was determined ex vivo, with a metaphyseal pQCT scan of the proximal tibia and defined as the inner 45% of the total cross-sectional area. Cortical bone parameters were determined ex vivo with a mid-diaphyseal pQCT scan of the femur.

Generation of Stable ERα and ERβ Reporter Cell Lines. Generation of stable human embryonic kidney 293 cells (American Type Culture Collection no. CRL 1573) expressing human ERα and human ERβ and the pΔERE2-ALP reporter vector has been described previously (Barkhem et al., 1998). All cell lines were cultured routinely at 37°C in humidified chambers at 5% CO_2 in minimal essential medium (phenol red-free) supplemented with 10% FCS and 2 mM L-glutamine.

Assay Procedure for Hormonal Effects on 293/hERα and 293/hERβ Reporter Cells. Cells (25×10^3 per well) were seeded onto 96-well culture plates in 100 μl of Coon's/F12 (phenol red-free) supplemented with 10% FCS (stripped twice using dextran-coated charcoal) and 2 mM L-glutamine. Twenty-four hours later, conditioned medium was replaced with 100 μl Coon's/F12 supplemented with 1% FCS (stripped twice using dextran-coated charcoal), 2 mM L-glutamine, gentamicin (50 μg/ml), and hormonal substances as indicated in the figure legends. In all experiments, cells were exposed to hormones for 72 h before harvest and analysis for effect on reporter-gene expression. Triplicate determinations of reporter protein levels in the conditioned media for each concentration of compound were performed in all experiments.

Assay for Human Placental Alkaline Phosphatase. The level of alkaline phosphatase (ALP) expressed from the ΔERE2-ALP reporter vector in the stably transformed 293/hERα and 293/hERβ reporter cells was determined using a chemiluminescent assay as follows: a 10-μl aliquot of heat-treated (at 65°C for 30 min) conditioned cell-culture medium was mixed with 200 μl of assay buffer (10 mM diethanolamine, pH 10.0, 1 mM $MgCl_2$, and 0.5 mM CSPD) in white microtiter plates (Dynatech Labs, Chantilly, VA) and incubated at 37°C for 20 min before being transferred to a microplate-format luminescence counter (1450 Microbeta; PerkinElmer Wallac, Turku, Finland). The setting of the Microbeta was for a 1-s reading of each well. The ALP activity is expressed in luminescence counts per second, which is directly proportional to the level of ALP expressed from the cells.

Results

Tissue Specificity for the Effect of Estren Compared with the Effect of 17β-Estradiol. To determine the tissue specificity for the effect of estren compared with the effect of 17β-estradiol, ovx mice were treated with vehicle, estren, or 17β-estradiol (0.7 μg/mouse/day). Surprisingly, already at an estren dose of 75 μg/mouse/day, a clear effect was seen on the uterine weight (132 ± 28% over vehicle), which is in contrast to a previous study using a 70% higher dose of estren and demonstrating no uterine effect (Kousteni et al., 2002). The effects of estren on different estrogen-responsive tissues were then compared with the effects of a physiological dose of 17β-estradiol (Lindberg et al., 2002). Both estren and 17β-estradiol increased the uterine weight compared with vehicle in ovx mice (Fig. 1). The effect of estren on the uterine weight was 16% of the effect exerted by 17β-estradiol (Table 1). As expected, the weights of the gonadal and the retroperitoneal fat deposits were reduced by 17β-estradiol in ovx mice. In contrast, no effect on these fat deposits was seen by estren treatment (Fig. 2). It is well known that estrogen treatment decreases the weight of thymus (Marotti et al., 1984). Both 17β-estradiol and estren treatment decreased the thymus weight, and for this parameter, the effects of estren and 17β-estradiol were of the same magnitude (17β-estradiol, −59 ± 11%; estren, −42 ± 6% compared with vehicle) (Table 1). Both estren and 17β-estradiol increased the trabecular BMD, and the effect of estren on trabecular BMD was 22% of the effect exerted by 17β-estradiol (Fig. 3A and Table 1). In contrast, cortical bone parameters, including cortical bone mineral content (BMC), cortical cross-sectional area, and cortical thickness, were increased by 17β-estradiol but not by estren (Fig. 3B, Table 1, and data not shown).

The Effects of Estren Are Mediated Via Estrogen Receptors. Because previous in vitro data have indicated that the effect of estren is mediated via ERs or the AR with similar efficiency (Kousteni et al., 2001, 2002), we designed an experiment to determine whether the in vivo effects of estren can be mediated via both the ERs and the AR. The in vivo effects of estren and 17β-estradiol were investigated in WT and $ERα^{-/-}β^{-/-}$ mice. The regulatory effects of estren and 17β-estradiol on uterus, fat, trabecular bone, and cortical bone, observed in WT mice, were lost in $ERα^{-/-}β^{-/-}$ mice (Figs. 1–3). Thus, the in vivo effects of estren and 17β-estradiol on uterus weight and trabecular bone are mediated via ERs, and the AR cannot replace the ERs for the mediation of these effects (Figs. 1–3).

Estren has Transcriptional Activity. The in vivo experiments demonstrated that the effects of estren are mediated via ERs. To determine whether estren has any transcriptional activity mediated via ERs, the effect of estren on the activation of an estrogen-responsive element-driven reporter gene (ALP) was tested in vitro in human 293 kidney epithe-

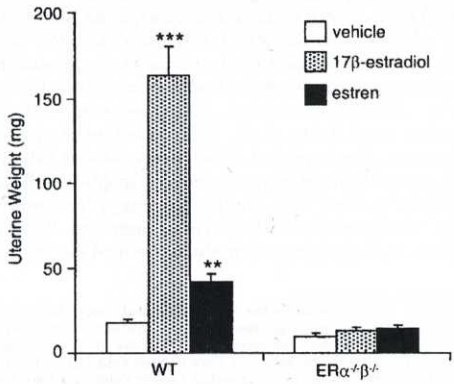

Fig. 1. Weight of uterus. Eleven-month-old female WT and $ERα^{-/-}β^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks (n = 7–8). Values given are means ± S.E.M. **, $p < 0.01$, ***, $p < 0.001$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

lial ERα (293/hERα)- and ERβ (293/hERβ)-expressing reporter cell lines (Barkhem et al., 1998). The responses of 293/hERα and 293/hERβ to estren are shown in Fig. 4, A and B, and expressed as the percentage of agonism of 17β-estradiol. Estren displayed a full agonistic effect on transcriptional activity with a degree of agonism similar to that of 17β-estradiol for both receptors (99% agonistic activity via ERα and 87% agonistic activity via ERβ compared with the activity of 17β-estradiol) (Fig. 4, A and B). As expected from previous ERα and ERβ binding studies (Kousteni et al., 2002), the transcriptional potency of estren was lower than that of 17β-estradiol in both the 293/hERα and the 293/hERβ cell lines (Fig. 4, A and B). The ER antagonist ICI 182,780 antagonized, in a dose-dependent manner, the effect of estren in both the 293/hERα and the 293/hERβ cells. The potency of ICI 182,780 in antagonizing estren-induced gene expression was similar in both reporter cell lines (IC_{50} values: 0.75 nM for ERα and 0.57 nM for ERβ) (Fig. 4, C and D). Thus, estren is a full agonist for the transcriptional activity mediated via both ERα and ERβ.

Discussion

Estrogen replacement therapy is beneficial for the treatment of postmenopausal bone loss but is reported to have undesirable effects on reproductive tissue and is associated with an increased risk of breast cancer (Riggs and Hartmann, 2003). SERMs having the beneficial effects of estrogen in bone with reduced side effects in breast and uterus have been developed. However, currently available SERMs are less potent in bone than estrogen replacement therapy (Delmas et al., 1997). Therefore, the recent description of estren as a potent stimulator of bone mass and strength without any side effects on reproductive organs has been very much appreciated (Kousteni et al., 2001, 2002). Furthermore, the described mode of action for the bone-sparing effect of estren is unique because it was reported to be a nongenomic effect involving functional and direct interactions with components of the Src/Shc/extracellular signal-regulated kinase signaling pathways. Extranuclear signaling by classic ERs and AR is supported by numerous reports describing interactions between sex-steroid receptors and components of the intracellular signaling machinery including phosphatidylinositol-3-phosphate kinase, Src, and Shc (Migliaccio et al., 2000; Simoncini et al., 2000; Duan et al., 2001; Kousteni et al., 2001). The recently described extranuclear mode of action of estren in bone has led to the definition of a novel class of mechanism-specific substances called ANGELS (Activator of NonGenotropic Estrogen-Like Signaling) (Manolagas et al., 2002). However, the present study indicates that estren rather, in a wider perspective, is a SERM with transcriptional activity.

The results of our present characterization of the effects of estren are in conflict with two previous publications from one research group describing the in vitro and in vivo effects of estren (Kousteni et al., 2001, 2002). In the in vivo study by Kousteni et al., it was found that estren had no effect on the uterine weight, whereas in the present study, using even a slightly lower dose of estren than in the previous study, a clear estren-induced increase in uterine weight was seen. In agreement with the previous study, the amount of trabecular bone was increased by estren in the present study. However,

TABLE 1

Tissue specificity for the effect of estren compared with the effect of 17β-estradiol on uterine weight, gonadal fat weight, thymus weight, trabecular BMD, and cortical BMC

Eleven-month-old female mice were ovariectomized and then treated for 4 weeks with vehicle, 17β-estradiol, or estren (n = 8). Values for the effect of estren compared with the effect of 17β-estradiol are given as the effect of estren divided by the effect of 17β-estradiol and are expressed as a percentage (equal to the percentage of estrogenic activity of estren) and as the relative tissue specificity for the estrogenic activity of estren when the effect on uterus is given as 1 (equal to the relative tissue specificity of estren).

Parameter	Estrogenic Activity of Estren	Relative Tissue Specificity of Estren (Uterus = 1)
	%	
Uterine weight	16.2	1
Gonadal fat weight	0	0
Thymus weight	71	4.4
Trabecular BMD	21.8	1.3
Cortical BMC	0	0

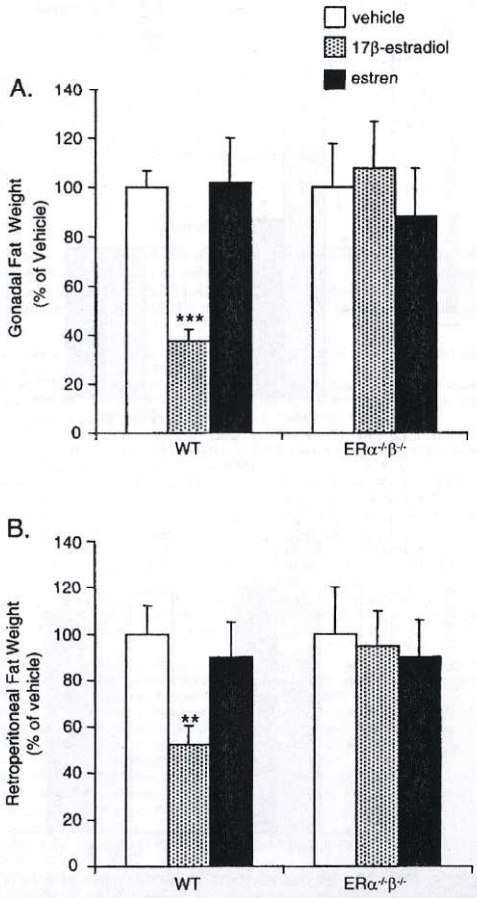

Fig. 2. Weight of gonadal (A) and retroperitoneal (B) fat deposits. Eleven-month-old female WT and $ERα^{-/-}β^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks (n = 8). Values are given as means ± S.E.M. **, $p < 0.01$, ***, $p < 0.001$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

in our study, the estrogen-like activity of estren was of the same magnitude on uterus as on trabecular bone, whereas in the previous study, a clear effect was seen on bone without any effect on uterus. It is difficult to explain the different results of the two studies. One may speculate that the 60-day slow-release treatment in the previous study was active during most of the time but not during the last few days of treatment. This would then result in bone, a slow-responding tissue, being preserved, with the uterus, a fast-responding tissue, no longer showing any effects of estren at the end of the treatment period. In contrast, in the present study, estren was given as subcutaneous daily injections during the whole experiment to ensure that the animals received the treatment until the final analysis.

In the present study, the effect of estren on several different tissues was investigated and compared with the effect of 17β-estradiol. It was demonstrated that estren exerts a relatively strong effect on thymus weight, a medium effect on uterus weight and trabecular bone, and no effect on fat mass or cortical bone. Thus, the degree of the estrogen-like activity of estren is tissue-specific.

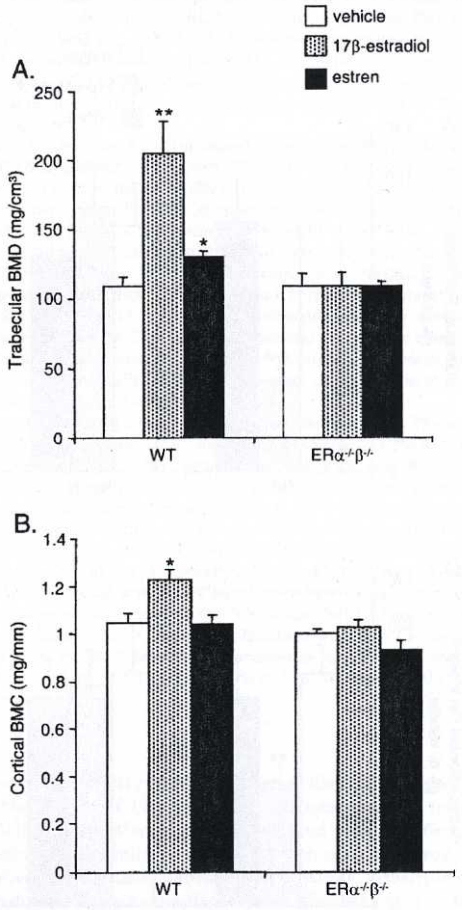

Fig. 3. A, trabecular volumetric BMD of the metaphyseal area of the proximal tibia measured using pQCT. B, cortical BMC as determined using a mid-diaphyseal pQCT scan of the femur. Eleven-month-old female WT and ERα$^{-/-}$β$^{-/-}$ mice were ovariectomized and then treated with vehicle, 17β-estradiol (0.7 μg/mouse/day), or estren (75 μg/mouse/day) for 4 weeks (n = 8). Values are given as means ± S.E.M. **, $p < 0.01$, *$p < 0.05$ versus vehicle, Student's t test followed by post hoc analysis according to Bonferroni test.

Previous in vitro data have indicated that the effect of estren might be mediated via ERs or the AR with similar efficiency (Kousteni et al., 2001, 2002). Here, we demonstrate in vivo that the effects of estren on trabecular bone and uterus are mediated via ERs and that the AR cannot replace the ERs in mediating these effects. We also demonstrate that the trabecular bone-sparing effect of 17β-estradiol is mediated only via ERs and not via the AR. Furthermore, the bone-sparing effect of 5α-dihydrotestosterone-induced AR stimulation is not dependent on the ERs (Movérare et al., 2003). Therefore, in contrast to what has recently been concluded from in vitro studies (Kousteni et al., 2001), there is no cross-reactivity between ERs and AR for the mediation of the trabecular bone-sparing effect of sex steroids, and the effect of estren in vivo is only mediated via ERs. Unfortunately, because of a complex breeding procedure of the ER-inactivated mice, no sham-operated control group was included in the present study. However, we have in a previous study seen that the 17β-estradiol dose given to the ovx mice in the present study results in sham-operated control levels for the different estrogen-responsive parameters analyzed in the present study (data not shown).

Because of an in vitro finding that estren did not induce C3 transcription in ERα-transfected HeLa cells, it was postulated that estren has no transcriptional activity and that all of its effects must be nongenomic (Kousteni et al., 2001). In contrast, using another well-established in vitro system of human 293 kidney epithelial ERα- and ERβ-expressing reporter cell lines (Barkhem et al., 1998), we demonstrated that estren displays a full agonistic effect on transcriptional activity with a degree of agonism similar to that of 17β-estradiol for both ERα and ERβ. The specificity of this effect was confirmed by the result that the ER antagonist ICI 182,780 antagonized, in a dose-dependent manner, the effect of estren on the transcriptional activity mediated via both receptors. Thus, estren has the capacity to exert genomic effects via both ERα and ERβ. In addition, we have also monitored the genomic response to estren in the human endometrial carcinoma cell line Ishikawa (Littlefield et al., 1990) by analyzing the expression of the endogenous alkaline phosphatase gene. Similar to the response in the genetically engineered 293 ERα and ERβ reporter cell lines, estren generated an agonist response that was totally blunted by the pure ER antagonist ICI 182,780 (data not shown), supporting the notion that estren exerts ER-mediated genomic effects.

The conflicting results in the previous study using HeLa cells compared with the present study using 293 kidney epithelial cells might depend on the choice of promoter and/or the choice of cell line. It is well known that the effect of ERs is exclusively mediated via AF-2 in HeLa cells (Berry et al., 1990; Metivier et al., 2000), whereas in the 293 kidney epithelial cells, the activity of the ERs is dependent on the function of both AF-1 and AF-2. The lack of transcriptional activity of estren in HeLa cells might indicate that the agonist effect of estren on ERs requires functionality of both AF-1 and AF-2 (Berry et al., 1990; Metivier et al., 2000). We do not doubt that estren, in analogy to 17β-estradiol, also

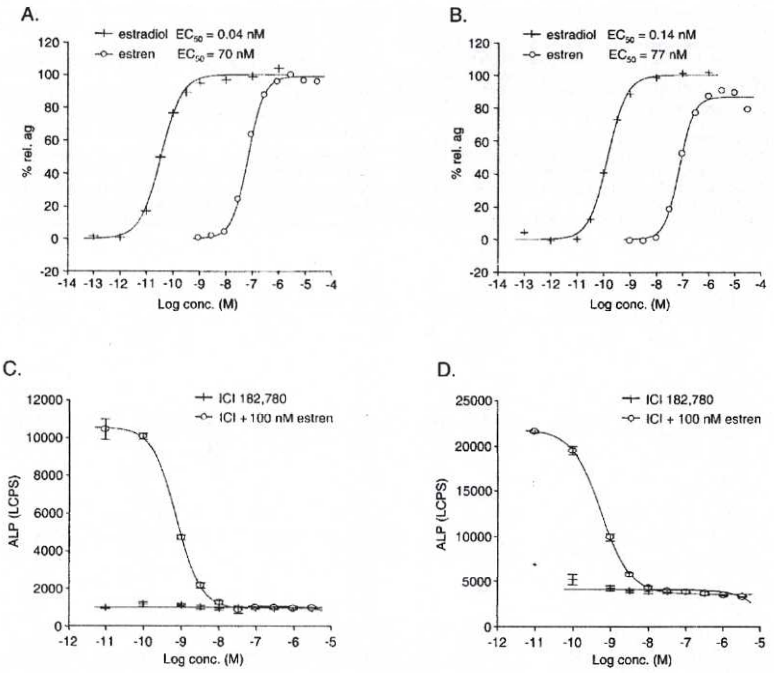

Fig. 4. Effect of estren and 17β-estradiol on transcriptional activity. Response to increasing concentrations of 17β-estradiol and estren in the 293/hERα (A) and 293/hERβ (B) reporter cell lines. The level of ALP reporter protein expressed was analyzed 72 h after the addition of the ligands. Antagonist response to increasing concentration of ICI 182,780 in the presence or absence of estren (100 nM) in 293/hERα (C) and 293/hERβ (D) reporter cell lines. The response values for each concentration of ligand are the means of triplicate determinations with the means ± S.D. for each value indicated.

elicits nongenomic responses, but our data clearly indicate, in contrast to the data from the study by Kousteni et al. (2002), that estren also has typical genomic effects. Thus, our data suggest that the biological effect of estren cannot solely be explained by a nongenomic mechanism but that genomic mechanisms of estren also have to be considered.

In conclusion, our results demonstrate that estren is a SERM with effects on uterus, trabecular bone, and thymus but without major effect on fat or cortical bone. The effects of estren on bone and uterus are mediated via ERs, and the AR cannot replace the ERs for these effects. Furthermore, it is clear that estren has the capacity to exert genomic effects mediated via ERs.

Acknowledgments

We thank Anette Hansevi and Maud Petersson for excellent technical assistance. We also thank the SWEGENE Center for Bio-Imaging for technical support regarding image analysis.

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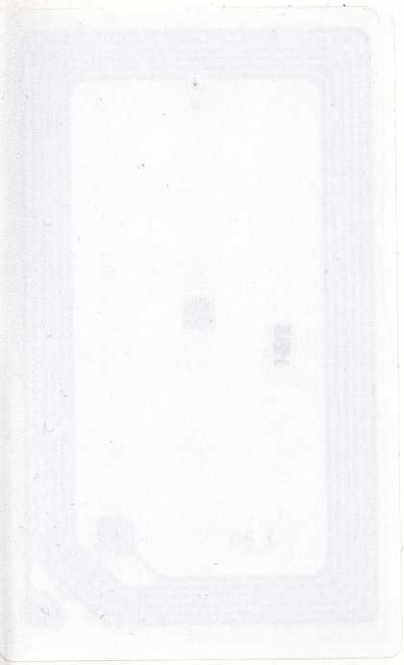

ISBN 91-628-6688-5