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PII: S0303-7207(08)00119-6
DOI: doi:10.1016/j.mce.2008.03.003
Reference: MCE 6844

To appear in: *Molecular and Cellular Endocrinology*

Received date: 30-1-2008
Revised date: 10-3-2008
Accepted date: 10-3-2008

Please cite this article as: Hertrampf, T., Schleipen, B., Velders, M., Laudenbach, U., Fritzemeier, K.H., Diel, P., Estrogen receptor subtype-specific effects on markers of bone homeostasis, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2008.03.003

Estrogen receptor subtype-specific effects on markers of bone homeostasis

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Abstract

To further elucidate the processes involved in the physiology of bone-protection by estrogens, ovariectomized (OVX) rats were treated subcutaneously with 17β-estradiol, the ERα-specific agonist (16α-LE2) and the ERβ-specific agonist (8β-VE2). OVX and intact animals served as controls. Biomarkers of bone-formation (osteocalcin (OC), osteopontin (OPN)) and bone-resorption (telopeptides of collagen type I (CTx), pyridinoline cross-links (Pyd)) were quantified. Bone mineral density was measured by computed tomography.

OVX-induced bone loss could be antagonized by subcutaneous administration of 17β-estradiol and 16α-LE2. Serum levels of CTx, OC and OPN were significantly elevated in OVX compared to intact animals and reduced by 17β-estradiol and 16α-LE2. Treatment of OVX rats with 8β-VE2 did not affect BMD or bone-marker serum levels.

Taken together, the complex expression pattern of bone-markers in OVX rats following subcutaneous administration of ER subtype-specific agonists indicates that 17β-estradiol exerts its bone-protective effects by modulating the activity of osteoclasts and osteoblasts via ERα.

Key words: estrogen receptor alpha, estrogen receptor beta, estrogen receptor subtype-specific agonists, biochemical bone markers, bone mineral density, estrogens

Abbreviations:

16α-LE2	ERα-specific agonist
8β-VE2	ERβ-specific agonist
BALP	bone specific alkaline phosphatase
b. wt.	body weight
CTx	C-terminal telopeptide
d	day
DAI	Daidzein
Dpd	desoxipyridinoline cross-links
E_2	17β-estradiol/ group substituted with a low dose of E_2
ELISA	enzyme linked immunosorbent assay
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
Fas	Faslodex
GEN:	Genistein
HRT	hormone replacement therapy
IL-6	interleukin-6
IDD	isoflavone depleted diet
NTx	N-terminal telopeptide
OC	osteocalcin
OPN	osteopontin
OPG	osteoprotegerin
pQCT	peripheral quantitative computed tomography
Pyd	pyridinoline cross-links

RAL	raloxifene
RANK	receptor activator of nuclear factor kappaB
RANKL	receptor activator of nuclear factor kappaB ligand
s.c.	subcutaneous
SERM	selective estrogen receptor modulator
TAM	tamoxifen
TRAP5b	tartrate-resistant acid phosphatase isoform 5b

Introduction

In the estrogen-deficient state, such as menopause, the balance between bone resorption and bone formation shifts towards increased levels of bone resorption. Hormone replacement therapy (HRT), if started soon after the onset of menopause, is effective for reducing or reversing postmenopausal bone loss (Lindsay et al. 1976, Fitzpatrick 2006). Besides its use for treatment of postmenopausal symptoms, potential benefits of HRT include the prevention of cardiovascular disease and dementia (Stevenson 2004). SERMs like Tamoxifen (TAM) and Raloxifene (RAL) are known to preserve bone mineral density (BMD) and act in breast tissue as estrogen antagonists (Diez-Perez 2006). Unfortunately, in postmenopausal women who take TAM the risk of developing endometrial cancer increases (Jordan 2008). Hence, an urgent need exists to develop new pharmaceutical agents that mediate the bone-protective effects of estrogens without increasing the risk of developing breast and endometrial cancer.

The anabolic activity of osteoblasts and the pathways through which these bone cells activate osteoclasts are affected by estrogens (Girasole et al. 1992) and selective estrogen receptor (ER) modulators (SERMs) (Taranta et al. 2002). Moreover, estrogens have the ability to decrease the differentiation of osteoclast progenitor cells (Sato et al. 2001, Sorensen et al. 2006) and to inhibit the bone-resorbing activity of terminally differentiated osteoclasts (Lerner 2006). Since both ER subtypes (ERα and ERβ) are expressed in bone tissue (Bland 2000, Bord et al. 2001, Krassas & Papadopoulou 2001) it can be assumed that estrogens and SERMs mediate their bone-remodelling effects by directly binding to ERs expressed within bone cells (Bryant et al. 1999, Diez 2000). Moreover, it has to be taken into consideration that skeletal effects of estrogen are not only mediated by classical but also by non-

classical ER pathways (Syed et al. 2005). Although bone cells express both ER subtypes, *in vitro* studies (Zaman et al. 2000) and those conducted *in vivo* with ER knockout mice (Windahl et al. 1999, Lee et al. 2003) or ER subtype-specific ligands (Hertrampf et al. 2007) have shown that ERα but not ERβ is required for bone-protection by estrogens.

It is well described that bone homeostasis depends on the balance of two different processes: formation and mineralization of bone matrix through osteoblasts and removal of mineralized bone by osteoclasts (Buckwalter et al. 1996, Hadjidakis & Androulakis 2006). Trabecular rather than cortical bone is more frequently remodelled, which explains why metabolic bone diseases such as osteoporosis are mainly observed in bones with comparatively large amounts of trabecular bone (Lerner 2006). Activation and induction of differentiation of osteoclast progenitor cells by osteoblasts is mediated through the receptor activator of nuclear factor kappaB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) signalling pathway (Lerner 2006, Blair et al. 2007). In this context, it was shown that inactivation of RANKL by a specific antibody leads to profound and prolonged inhibition of bone-resorption in postmenopausal women (Hamdy 2007). Furthermore, ERα but not ERβ is known to be involved in the regulation of the ratio of OPG and RANKL (Lindberg et al. 2001b).

The activity of osteoclasts and osteoblasts can be monitored by measuring sensitive and specific circulating markers (Allen 2003). Classic markers for bone formation are bone-specific alkaline phosphatase (BALP) (Deftos et al. 1991), osteocalcin (OC) (Thiede et al. 1994), osteopontin (OPN) (Butler 1989, Sodek et al. 2000, Morinobu et al. 2003) and collagen propeptides (PICP,PINP) (Eberling et al. 1992), while serum and urinary levels of telopeptides of collagen type I (CTx, NTx), pyridinoline cross-links (Pyd, Dpd) and tartrate-resistant acid phosphatase isoform 5b (TRAP5b)

(Janckila et al. 2001) positively correlate with bone resorption, with high levels indicating excessive osteoclastic activity (Coleman 2002, Hanson et al. 1992).

In this context, the aim of the present study was to further elucidate the ER-subtype specific molecular mechanisms involved in bone protection. Therefore, OVX rats, a suitable animal model for studying processes underlying osteoporosis (Kalu 1991), were treated subcutaneously (s.c.) with ER subtype-specific agonists for ERα (16α-LE2) or ERβ (8β-VE2) over a period of three weeks. Intact (SHAM) animals, vehicle-treated OVX animals on an isoflavone depleted diet (IDD) and OVX rats treated s.c. with 17β-estradiol (E_2) served as controls.

To elucidate the mechanism(s) responsible for bone-protection by estrogens *in vivo*, bone mineral density (BMD) was measured by peripheral computed tomography (pQCT) and serum levels of several markers for bone formation (OC) and bone resorption (CTx, Pyd) as well as the extracellular glycoprotein OPN, secreted by osteoblasts, were assayed using commercial ELISA kits.

Material and Methods

Substances:

17β-Estradiol (Estra-1,3,5(10)-trien-3,16α,17 β-diol), was provided by Sigma-Aldrich (Deisenhofen, Germany). The specific estrogen receptor agonists for ERα (16α-LE2, 3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone) and ERβ (8β-VE2, 8-vinylestra-1,3,5 (10)-triene-3,17β-diol) (Fig.1) were provided by the Bayer Schering Pharma AG (Berlin, Germany) and the pure antiestrogen Faslodex (ICI 182,780) was provided by AstraZeneca (Wedel, Germany).

Diet:

Animals had free access to a diet low in phytoestrogen content (IDD) (SSniff GmbH, Soest, Germany) and water. The isoflavone contents of the IDD (Daidzein < 10µg/g, Genistein < 10µg/g) have been determined by HPLC analysis in a previous study from our laboratory (Hertrampf et al. 2006).

Animals:

Female Wistar rats aged 8 weeks (125-150g) were obtained from Janvier (Janvier, Le Genest St Isle, France) and were maintained under controlled conditions of temperature (20°C ± 1, relative humidity 50-80%) and illumination (12 h light, 12 h dark). All animal experiments were approved by the Committee on Animal Care and complied with accepted veterinary medical practice.

Animal treatment and tissue preparation:

Adult animals were ovariectomized (OVX) or SHAM operated at the age of 12 weeks, weighing 200-220g. After 14 days of endogenous hormonal decline the animals were

treated with the test compounds or vehicle for 3 weeks. The animals were randomly allocated to treatment and vehicle groups (n = 6). E_2 (4µg kg^{-1}b.wt d^{-1}), 16α-LE2 (10µg kg^{-1}b.wt d^{-1}) and 8β-VE2 (100µg kg^{-1}b.wt d^{-1}) were dissolved in dimethylsulfoxide (DMSO) (200µl kg^{-1}b.wt d^{-1}) and corn oil (800µl kg^{-1}b.wt d^{-1}) for s.c. administration. For isotype-specific ER activation, we used the selective ER-agonists 16α-LE2 and 8β-VE2 (Fig.1). Because these compounds activate both receptors at higher concentrations (Hegele-Hartung et al. 2004), doses of 10 µg kg^{-1}b.wt d^{-1} (16α-LE2) and 100 µg kg^{-1}b.wt d^{-1} (8β-VE2) were chosen respectively. For these doses activation and subsequent signaling through either ERα or ERβ respectively can be anticipated (Hegele-Hartung et al. 2004, Hillisch et al. 2004). Animals were sacrificed by decapitation after light anesthesia with CO_2 inhalation. Uteri were prepared free of fat and the wet weights were determined.

Determination of bone mineral density

The right tibiae were snap frozen in liquid nitrogen. BMD was measured by peripheral quantitative computed tomography (pQCT) (XCT Research SA+, StraTec Medizintechnik, Pforzheim, Germany). Trabecular density (measured by density mode, ROI at 7.5% of bone length), cortical density (ROI at 50% of bone length) and total density (ROI at 7.5% and 50% of bone length) of the tibiae were measured *ex vivo* at the end of the study after 3 weeks of treatment.

Quantification of bone markers

Serum concentrations of the bone formation marker osteocalcin (OC) (Thiede et al. 1994) were assayed using a commercial rat ELISA kit (Metra OC P, Quidel Corporation, San Diego, CA).

Serum levels of telopeptides of CTx and Pyd, that correlate with bone resorption, with high levels indicating excessive osteoclastic activity (Coleman 2002, Hanson et al. 1992) were also analysed using commercial ELISA kits (Serum CrossLaps, Nordic Bioscience, Herlev, Denmark; Metra Serum Pyd, Quidel Corporation, San Diego, CA). OPN EDTA-plasma levels were also quantified by ELISA (Immuno-Biological Laboratories, Hamburg, Germany). All ELISAs were performed using a plate washer (HydroFlex Platform, Tecan GmbH, Crailsheim, Germany).

Statistical analysis

Statistical analyses were performed using the SPSS Statistical Analysis System, Version 12.0.

All data are expressed as arithmetic means with their standard errors. Statistical significance of differences was calculated using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey HSD test where appropriate. Statistical tests were used for comparisons between groups and statistical significance was established at $P<0.05$.

Results

OVX resulted in a reduction (6-fold) of uterine wet weights compared to SHAM animals (Fig.2 A). Treatment of OVX animals with 17β-estradiol (E_2) led to a stimulation of uterine wet weights (5-fold) relative to untreated OVX animals. In contrast to s.c. administration of 16α-LE2 (4.5-fold), uterine wet weights were not affected by application of 8β-VE2 (Fig.2 A).

After 3 weeks, body weights of OVX animals were significantly elevated compared to SHAM animals (Fig.2 B). OVX-induced body weight gain could be antagonized by s.c. administration of E_2 and 16α-LE2, but was not significantly different to OVX in the group treated with 8β-VE2 (Fig.2 B).

Trabecular BMD was significantly reduced in tibiae of OVX animals compared to intact (SHAM) rats (Fig.2 C). In contrast to application of 8β-VE2, s.c. administration of E_2 as well as treatment with 16α-LE2 over a period of three weeks equally resulted in an increased trabecular BMD compared to OVX (Fig.2 C).

Besides reduced trabecular BMD in the tibia, serum levels of the collagen degradation product CTx, was signifficantly elevated in OVX animals compared to SHAM animals (Fig. 3 B). However, the induced levels of the bone resorption marker Pyd detected in OVX animals were not significantly different from SHAM animals (Fig. 3 D). Moreover, serum levels of the bone formation markers (OC and OPN) were significantly higher in OVX animals (Fig. 3 A and C). In line with E_2, but in contrast to treatment of OVX animals with 8β-VE2, application of 16α-LE2 resulted in significantly reduced serum levels of CTx, OC and OPN (Fig.3). Pyd serum levels tended to be reduced in SHAM and OVX animals treated with 16α-LE2 but

significance was only observed in the group substituted with E_2 relative to vehicle-treated OVX animals (Fig. 3 D).

Discussion

The aim of the present study was to examine ER subtype-specific effects on bone mineral density (BMD) and expression patterns of certain markers for bone resorption and bone formation in adult female Wistar rats. OVX rats, a suitable animal model for studying processes underlying post-menopausal osteoporosis (Kalu 1991), were treated s.c. with ER subtype-specific agonists for ERα (16α-LE2) or ERβ (8β-VE2). Intact (SHAM) and OVX rats on an isoflavone depleted diet (IDD), and OVX animals substituted with 17β-estradiol (E_2) served as controls.

The validity of our model system of menopause was confirmed by the analysis of classical biological endpoints for estrogenic action. For example, treatment with the respective compounds affected uterine wet weight in accordance with published literature (Pelzer et al. 2005, Hertrampf et al. 2007). 16α-LE2 stimulated uterine wet weights in a comparable manner to E_2 (Fig.2 A) (Hegele-Hartung et al. 2004) whereas treatment with 8β-VE2 did not result in a significant stimulation of the uterine wet weight (Hillisch et al. 2004).

Elevated energy intake and increased body weight are both known to be associated with reduced levels of estrogens in OVX rats (Heine et al. 2000). In line with previous findings from our laboratory, our data shows that OVX rats display a stronger increase of body weight compared to E_2 and SHAM rats mainly due to an increase in the percentage of total body fat (Lindberg et al. 2001a, Hertrampf et al. 2007). In contrast to treatment with 8β-VE2, application of 16α-LE2 resulted in an antagonization of body weight increase after OVX (Fig. 2 B). This is in agreement with studies using male and female ERα knockout mice (ERKO). In these animals lipid metabolism-related gene expression in adipose tissue and body fat composition are influenced by ERα-specific signalling (Ohlsson et al 2000, Heine et al. 2000,

Mueller et al. 2001). Our data demonstrate that the estrogen-dependent modulation of body weight and composition in wild type animals with natural ratios of both ER subtypes is also mainly mediated via ERα (Fig.2 B) (Lindberg et al. 2001a, Hertrampf et al. 2007).

According to previous findings from our laboratory, treatment with 16α-LE2, E_2 but not 8β-VE2 resulted in a significant increase of trabecular BMD compared to OVX animals (Fig.2 C), an effect that can be antagonized by the pure antiestrogen Fas (Hertrampf et al. 2007). These data are in agreement with studies performed in knockout mice: application of E_2 did not prevent the development of osteoporosis in ERαKO female (Lindberg et al. 2001a, Lindberg et al. 2002a) and male mice (Lindberg et al. 2002b), indicating that the bone-protective effects of E_2 are primarily mediated by ERα. Further, ERα gene polymorphisms are associated with osteoporosis in human populations (Gómez et al. 2007).

On the other hand differentiation of bone marrow cells lacking ERα, can be stimulated by E_2 relatively to the same extent as in wild type cells (Parikka et al. 2005), indicating ERβ to be involved in bone marrow cell differentiation. Opposing effects were observed for longitudinal bone growth after activation of ERα and ERβ (Windahl et al. 1999, Lindberg et al. 2001a), suggesting a repressive function for ERβ in the regulation of bone growth during adolescence.

Because the impact of the two ER subtypes on bone metabolism is not fully understood, this study aimed to examine the impact of ER subtype-specific agonists on physiological serum markers of bone resorption and bone formation.

All tested serum markers were significantly elevated in OVX compared to SHAM and E_2 animals (Fig.3). In the OVX group substituted with 16α-LE2, serum levels of OC,

OPN and CTx were significantly lower than in the OVX group, while Pyd levels only tended to be reduced (Fig.3).

OC, one of the very few molecules exclusively produced by osteoblasts, is a widely used marker for bone formation (Lerner 2006). In postmenopausal osteoporosis bone resorption as well as bone formation are increased (Lerner 2006). In line with this are our current findings showing that both bone formation markers like OC and OPN as well as excretion levels of collagen degradation products (CTx, Pyd) are elevated in OVX rats compared to SHAM animals, and decreased in OVX animals treated with E_2 and 16α-LE2 (Fig.3). In contrast to treatment with E_2 and 16α-LE2, neither serum levels of OC and OPN nor those of CTx and Pyd were affected by 8β-VE2 application compared to OVX (Fig.3).These results indicate that the activation of ERα is mainly responsible for the estrogenic stimulatory effects on osteoblastic bone-formation and inhibitory effects on osteoclastic bone-resorption. This observation agrees with the results of Lindberg et al. (2001b) showing that ERα but nor ERβ is involved in the regulation of the OPG/RANKL ratio and serum levels of interleukin-6 (IL-6) and tartrate-resistant acid phosphatase 5b (TRAP5b). Moreover, it was shown that estrogens regulate the life span of mature osteoclasts by inducing apoptosis via ERα (Nakamura et al. 2007), and that ERα is required for proliferation of osteoblast-like cells *in vitro* (Lee et al. 2003).

Taken together, the trabecular BMD data of the present study and the complex secretion patterns of certain markers for bone-resorption and bone-formation indicate that, in contrast to 8β-VE2, 16α-LE2 mediates bone-protective effects by inhibiting OPN and OC secretion in osteoblasts. In addition to reduced OC and OPN serum levels, application of 16α-LE2 but not 8β-VE2 resulted in an inhibition of bone-resorption, represented by reduced serum levels of CTx and Pyd.

The results of the present study lead us to the conclusion that bone-resorption by osteoclasts and bone-formation by osteoblasts are both influenced by estrogens and estrogenic compounds via ERα but not ERβ. Further studies should be conducted to elucidate if ERβ antagonizes ERα-specific signalling in bone or is more likely responsible for bone cell differentiation.

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Fig.:1

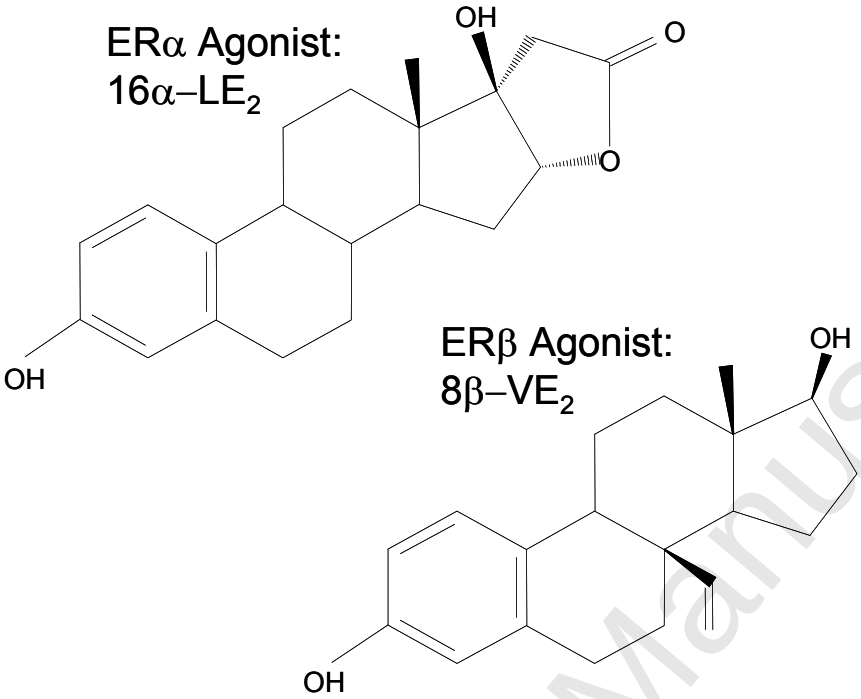

Fig.:2

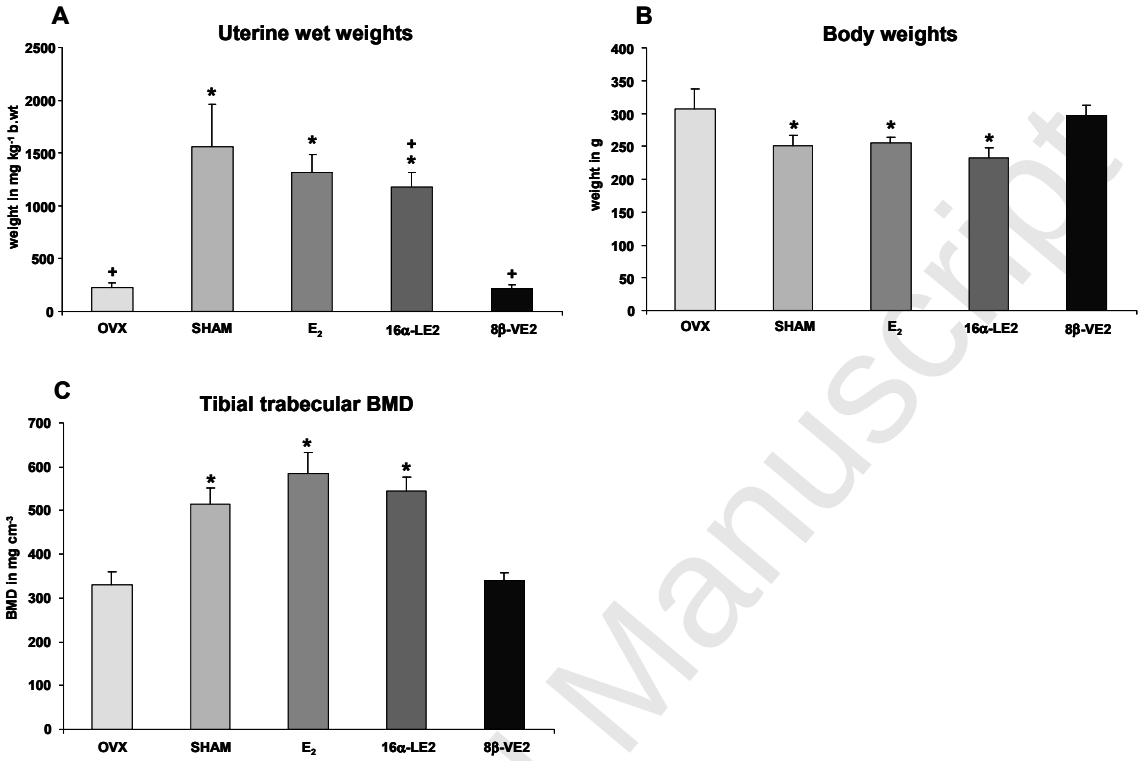

Fig.:3

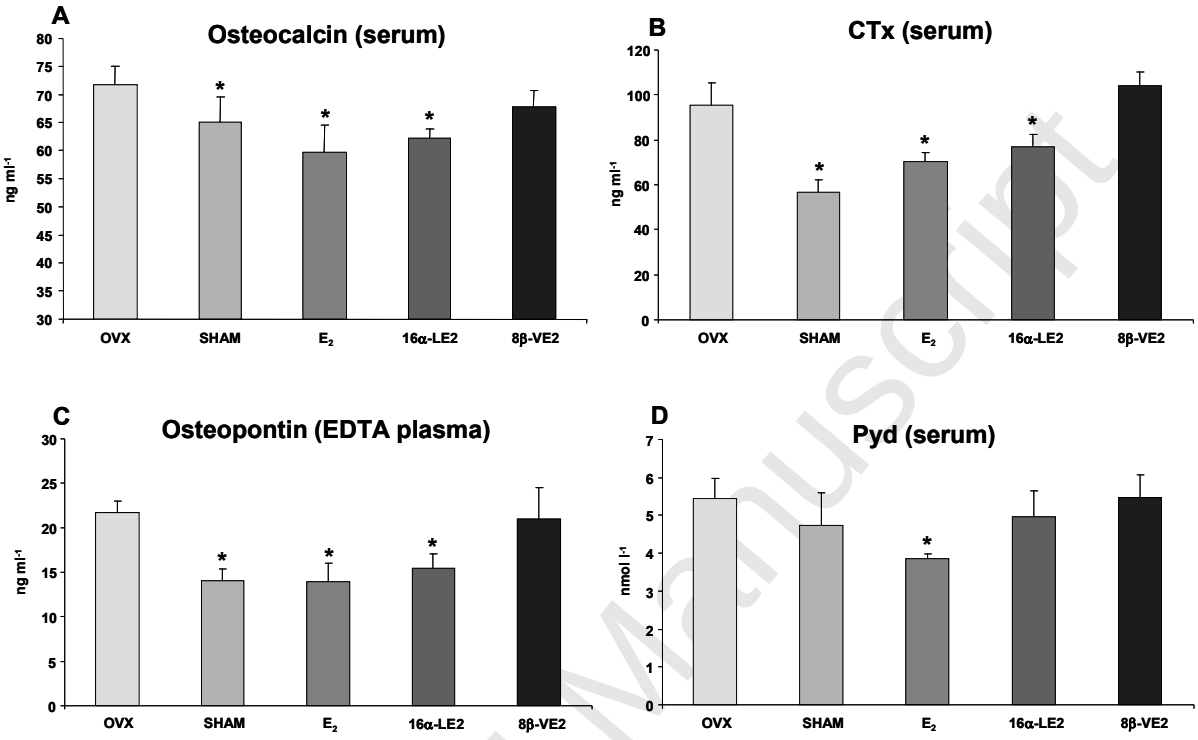

Figure legends

Fig.1 ER subtype-specific agonists.

Chemical structures of the specific ER agonists for ERα: 16α-LE_2 and ERβ: 8β-VE_2.

Fig.2 Physiological end points.

Uterine wet weights (A), Body weights (B) and trabecular bone mineral density (C) after 3 weeks of treatment.

Abbreviations: SHAM = intact, SHAM operated animals fed IDD, E_2 = OVX group treated s.c. with 17β- estradiol (4μg kg^{-1}b.wt d^{-1}), OVX = vehicle-treated OVX group fed IDD, 16α-LE_2 = OVX group treated s.c. with 16α-LE_2 (10μg kg^{-1}b.wt d^{-1}), 8β-VE_2 = OVX group treated s.c. with 8β-VE_2 (100μg kg^{-1}b.wt d^{-1}). Experimental conditions and treatment procedures are explained in detail in Materials and Methods.

* Denotes values significantly different from ovariectomized group (OVX)

P < =0.05, ANOVA, n=6

$^{+}$ Denotes values significantly different from intact group (SHAM)

P < =0.05, ANOVA, n=6

Fig.3 Bone markers.

Abbreviations: SHAM = intact, SHAM operated animals fed IDD, E_2 = OVX group treated s.c. with 17β- estradiol (4µg kg^{-1}b.wt d^{-1}), OVX = vehicle-treated OVX group fed IDD, 16α-LE_2 = OVX group treated s.c. with 16α-LE_2 (10µg kg^{-1}b.wt d^{-1}), 8β-VE_2 = OVX group treated s.c. with 8β-VE_2 (100µg kg^{-1}b.wt d^{-1}). Experimental conditions and treatment procedures are explained in detail in Materials and Methods.

* Denotes values significantly different from ovariectomized group (OVX)

P < =0.05, ANOVA, n=6