Tissue-selective effects of continuous release of 2-hydroxyestrone and 16α-hydroxyestrone on bone, uterus and mammary gland in ovariectomized growing rats

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Abstract

2-Hydroxyestrone (2-OHE_1) and 16α-hydroxyestrone (16α-OHE_1) have been reported to be risk factors for negative bone balance and breast cancer, respectively. The roles of these two metabolites of estrone as estrogen agonists or antagonists with respect to estrogen target tissues, or both, are poorly defined. The purpose of this study was to characterize metabolite and tissue-specific differences between the actions of hydroxylated estrones on selected reproductive and non-reproductive estrogen target tissues in growing rats. First, the effects of ovariectomy were determined. Ovariectomy had the expected effects, including increases in all dynamic bone measurements at the proximal tibial epiphysis, without induction of bone loss. Second, ovariectomized growing rats were continuously treated for 3 weeks with 2-OHE_1, 16α-OHE_1, 17β-estradiol (E_2), a combination of E_2 and 2-OHE_1 (E_2+2-OHE_1), or a combination of E_2 and 16α-OHE_1 (E_2+16α-OHE_1), using controlled release subcutaneous implanted pellets containing 5 mg 2-OHE_1, 5 mg 16α-OHE_1, 0·05 mg E_2 or placebo. E_2 reduced body weight gain and radial and longitudinal bone growth as well as indices of cancellous bone turnover, and increased serum cholesterol, uterine wet weight and epithelial cell height, and proliferative cell nuclear antigen labeling in mammary gland. The hydroxylated estrones did not alter uterine wet weight and 16α-OHE_1 antagonized the E_2-stimulated increase in epithelial cell height. 2-OHE_1 had no effect on cortical bone, whereas 16α-OHE_1 was an estrogen agonist with respect to all cortical bone measurements. 16α-OHE_1 also behaved as an estrogen agonist with respect to serum cholesterol and cancellous bone measurements. 2-OHE_1 had no effect on most E_2-regulated indices of cancellous bone growth and turnover, but was a weak estrogen agonist with respect to mineral apposition rate and bone formation rate. Neither estrogen metabolite influenced body weight gain. Third, weanling rats were treated for 1 week with vehicle, E_2 (200 μg/kg per day) or 16α-OHE_1 (30, 100, 300, 1000 and 3000 μg/kg per day) to confirm uterotropic effects of daily subcutaneous (s.c.) administration of 16α-OHE_1. 16α-OHE_1 increased uterine weight in a dose–response manner to values that did not differ from rats treated with E_2. We conclude that the estrogen metabolites 2-OHE_1 and 16α-OHE_1 have target tissue-specific biological activities which differ from one another as well as from E_2. These findings add further support to the concept that there are several classes of estrogens with distinct biological activities. Furthermore, differences in the route of administration could influence the tissue specificity of estrogen metabolites.

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Introduction

Although 17β-estradiol (E_2) exerts diverse biological effects in reproductive and non-reproductive tissues by direct interaction with a nuclear receptor, some actions of estrogen may result from a conversion of parent hormone to its metabolites (Martucci & Fishman 1993, Turner *et al.* 1994). E_2, in the presence of 17β-dehydrogenase, is metabolized to estrone (E_1), which is primarily hydroxylated in liver at either C-2 to form 2-hydroxyestrone (2-OHE_1) or C-16α to form 16α-hydroxyestrone (16α-OHE_1) (Vermeulen *et al.* 1976, Schneider *et al.* 1982).

2-OHE_1, which is sometimes referred to as the good estrogen, has been shown to have very weak estrogenic activity, and in some experimental systems it has been shown to have antagonistic activity (Vandewalle & Lefebvre 1989). In contrast, 16α-OHE_1 is considered to

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be an estrogen agonist, has low affinity for sex hormone binding globulin (Fishman & Martucci 1980) and binds with moderate affinity to the estrogen receptor (Swaneck & Fishman. 1988).

Estrogenic or antiestrogenic properties of estrogen metabolites may be associated with the development of breast cancer. Fishman and colleagues reported that 16α-OHE_1 covalently bound the estrogen receptor and induced breast cancer, whereas 2-OHE_1 primarily inhibited cell growth (Schneider *et al.* 1984, Swaneck & Fishman. 1988). Furthermore, 2-OHE_1 partially antagonized the stimulatory effect of E_2 on growth (Vandewalle & Lefebvre 1989). However, other experiments suggest that 2-OHE_1 is an estrogen agonist. 2-OHE_1 and 16α-OHE_1 increased cell proliferation in MCF-7 and T47D breast cancer cells and the induced mitogenic activity was inhibited by the estrogen antagonists, tamoxifen and ICI-182780 (Gupta *et al.* 1998). Thus there is no consensus as to the effects of 2-OHE_1 on the growth of breast cancer cells in culture, and no study has established a direct cause-and-effect relationship between the continuous presence of estrogen metabolites in the circulation and the incidence of breast cancer.

Our understanding of the biological actions of estrogen metabolites is primarily based on a very limited number of studies performed on reproductive tissues. The influence of estrogen metabolites on bone has only recently been investigated. Hodge and coworkers (1995) found that there was an association between 2-hydroxylation of E_1 and bone density in early postmenopausal women. In particular, urinary 2-OHE_1 was negatively correlated with vertebral bone density and had a tendency to increase in women with a family history of osteoporosis. Furthermore, African-American women, who had a greater bone mass and lower risk for developing osteoporosis compared with European-American women, have been reported to have a higher urinary 16α-OHE_1/2-OHE_1 ratio (Cauley *et al.* 1994, Taioli *et al.* 1996). More recent data suggested that women in the lowest quartile of urinary 2-OHE_1/16α-OHE_1 appeared to be protected from early postmenopausal bone loss (Leelawattana *et al.* 2000).

Studies in ovariectomized rats suggested that 16α-OHE_1 but not 2-OHE_1 is an estrogen agonist with respect to bone (Westerlind *et al.* 1998). The observed differences in activity of the two metabolites on bone metabolism could explain the reported differences in bone mass in women. Surprisingly, 16α-OHE_1 appeared to be a more potent estrogen agonist with respect to skeletal tissue than with respect to uterus and mammary gland (Westerlind *et al.* 1998). This apparent tissue-selective activity would be a beneficial characteristic for hormone replacement therapy. However, the effects of the metabolites on the response to 17β-estradiol were not investigated. Such competition studies are essential to determine whether the mechanism for the tissue-selective estrogen agonism/antagonism of 16α-OHE_1 is similar to that of tamoxifen and other selective estrogen receptor modulators (SERMs) (Turner *et al.* 1987). In addition, previous studies were not designed to determine whether 2-OHE_1 antagonizes the actions of E_2 on bone in a manner analogous to the effects reported in breast cancer cells (Schneider *et al.* 1984). Therefore, the aim of the present investigation in rats was to evaluate E_2 agonistic and antagonistic activities of 2-OHE_1 and 16α-OHE_1 in bone and other estrogen target organs. The goal was accomplished by continuous administration of the metabolites with implanted pellets in the presence and absence of a submaximal dose of 17β-estradiol. Growing animals were studied to investigate the effects of estrogen metabolites on the cellular processes that establish (growth) and maintain (modeling and remodeling) peak bone mass.

Materials and Methods

Animals

All animal procedures were approved by the institutional animal care and use committee at the Mayo Foundation (Rochester, MN, USA).

Sprague–Dawley rats (sham-operated (sham), ovariectomized (OVX) or weanling) were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN, USA). They were maintained on laboratory rat chow and water which were available *ad libitum* under a 12 h light:12 h darkness cycle in a temperature-controlled room.

Study I This experiment was performed to confirm that ovariectomy induced cancellous bone turnover without bone loss at the proximal tibial epiphysis. Female growing rats, weighing 180–200 g, were ovariectomized at 2 months of age. The rats were fluorochrome labeled (20 mg/kg) by juxta-tail vein injection of calcein (Sigma Chemical Co.) and demeclocycline (Sigma Chemical Co.) 9 and 2 days before they were killed, respectively. On day 27 after ovariectomy, all rats were weighed and anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (5 mg/kg). They were killed by cervical dislocation. Wet weights of uteri were recorded. The tibiae were removed, cleared of adhering tissue and fixed in 70% ethanol before being processed for static and dynamic bone histomorphometry.

Study II This experiment was performed to determine whether 2-OHE_1 or 16α-OHE_1 were partial estrogen agonists, antagonists, or both with respect to bone, mammary tissue and uterus. The rats were the same age as in study I. Six days after surgery, OVX rats were weighed and randomly divided into seven groups of animals : (i) baseline (n=10), (ii) vehicle (n=8), (iii) 2-OHE_1 (n=9), (iv) 16α-OHE_1 (n=9), (v) E_2+vehicle (n=9), (vi) E_2+2-OHE_1 (n=11), and (vii) E_2+16α-OHE_1 (n=9). The baseline group was killed on the day treatment was

initiated. The remaining groups were subcutaneously implanted with controlled release pellets (Innovative Research, Inc., Sarasota, FL, USA) containing placebo, 5 mg 2-OHE_1, 5 mg 16α-OHE_1, 0·05 mg E_2 or combination for 3 weeks. We have shown that the E_2 pellet implant, which delivers E_2 at a rate of approximately 15 μg/kg per day, maintains normal uterine weight and bone volume in OVX rats (Sibonga *et al.* 1998). Subsequent dose–response studies have shown that the 5 mg pellet delivers a robust but not maximal skeletal response to 16α-OHE_1 (unpublished data). The 5 mg 2-OHE_1 pellet was also chosen on the basis of previous studies demonstrating blood concentrations of the metabolite that greatly exceed physiological values (Westerlind *et al.* 1998).

The rats were fluorochrome-labeled with tetracycline (Sigma Chemical Co.) 1 day before starting treatment, calcein 9 days before they were killed, and demeclocycline 2 days before they were killed. On treatment day 21 (27 days after ovariectomy), blood was collected from abdominal aorta for measurement of serum cholesterol before death. The tibiae were removed as described in study I.

The uteri were quickly excised, weighed and fixed in 10% neutral buffered formalin for 24 h before being transferred to 70% ethanol until required for processing for conventional paraffin embedding for measurement of epithelial cell height. Left mammary glands were excised for quantitative proliferating cell nuclear antigen (PCNA) immunohistochemistry.

Study III This experiment was performed to verify that pulsatile administration of 16α-OHE_1 exhibited a uterotropic effect. Thirty-five weanling rats (3 weeks old) that were known to have low endogenous estrogen because of immaturity were randomly divided into seven groups with five animals per group. They received daily subcutaneous (s.c.) administration (0·1 ml) of (i) vehicle, (ii) 17β-estradiol (200 μg/kg per day) and (iii–vii) 16α-OHE_1 (30, 100, 300, 1000, and 3000 μg/kg per day) for 1 week. E_2 and 16α-OHE_1 were dissolved in 95% ethanol and diluted with water to 50% ethanol. On treatment day 8, the rats were killed as described in study I and the uteri were removed and weighed.

Bone histomorphometry

Bone histomorphometric parameters were measured with the Osteomeasure semi-automatic image analysis system (OsteoMetrics, Atlanta, GA, USA) as described elsewhere (Cavolina *et al.* 1997, Kidder & Turner 1998).

Cross sections of tibial diaphyses, approximately 150 μm thick, were cut at a site just proximal to the tibiofibular synostosis with a low-speed Isomet saw equipped with a diamond wafer blade (Buehler, Lake Bluff, IL, USA). They were ground on a roughened glass plate to a thickness of 20 μm and mounted on a glass slide with Eukitt mounting reagent (O Kindler, Germany). Two sections from each animal were examined at 4 × magnification. Cortical bone area (mm^2) was calculated by subtracting medullary area from cross section area. Periosteal perimeter (mm) was defined as the entire length surrounding the periosteal bone surface. Double-labeled perimeter (mm) was the periosteal perimeter labeled with double fluorochrome. Periosteal bone formation (mm^2/day) was calculated as the area of bone between the tetracycline and demeclocycline label divided by the labeling period of 20 days. Periosteal mineral apposition rate (μm/day) was calculated as the periosteal bone formation rate divided by the double-labeled perimeter.

The proximal metaphyses with attached epiphyses were dehydrated through an ascending series of ethanol, infiltrated, embedded in methylmethacrylate and sectioned at a thickness of 5 μm (Reichert-Jung Supercut 2050 microtome, Heidelberg, Germany). Sections were mounted for unstained static and dynamic determination of cancellous bone (study I and II). The consecutive sections were stained for acid phosphatase to determine osteoclast number (study II).

Parameters determined from unstained sections

Longitudinal growth rate in studies I and II was the mean distance between the calcein labeling front located in the primary spongiosa and the demeclocycline in the mineralizing growth plate cartilage divided by the labeling interval of 7 days.

Estrogen deficiency stimulates both bone modeling and remodeling in growing rats. An alteration in longitudinal bone growth during the first month after ovariectomy would affect trabecular bone at the metaphysis (Wronski *et al.* 1988, Sibonga *et al.* 1998). To exclude an effect of longitudinal growth on bone turnover, a standard sampling site with an area of 2·5 mm^2 was established within the epiphysis, 0·25 mm distal to cortical bone. Bone volume was defined as the percentage of tissue volume consisting of cancellous bone. Double-labeled surface, expressed as percent of bone surface, was the trabecular surface covered with calcein and demeclocycline labels. Mineral apposition rate (μm/day) was the mean distance between the calcein and demeclocycline label divided by the interval of 7 days. Bone formation rate was the product of the double-labeled surface and mineral apposition rate. It was normalized per bone surface referent (BFR/BS, expressed as $\mu m^3/\mu m^2$ per day), bone volume referent (BFR/BV, expressed as %/day) or tissue referent (BFR/TV, expressed as %/day)

Parameters determined from stained sections

Osteoclasts, expressed as the percent tissue area in the zone of vascular invasion, were identified as large multinucleated cells with an acid phosphatase-positive cytoplasm lying adjacent to cartilage matrix.

Table 1 Effect of ovariectomy on cancellous bone histomorphometric measurements. Values are means ± S.E.M.

	Sham ($n=8$)	**OVX** ($n=12$)
Parameters		
Bone volume/tissue volume (%)	37·31 ± 1·17	37·47 ± 1·29
Double-labeled surface/bone surface (%)	13·98 ± 1·21	19·84 ± 1·90*
Mineral apposition rate (μm/day)	0·86 ± 0·04	1·03 ± 0·05*
Bone formation rate (BFR)		
BFR/BS ($\mu m^3/\mu m^2$ per day)	0·12 ± 0·01	0·21 ± 0·03*
BFR/BV (%/day)	0·23 ± 0·02	0·43 ± 0·06*
BFR/TV (%/day)	0·09 ± 0·01	0·16 ± 0·03*

*$P<0·05$ compared with sham group.

Serum cholesterol

Blood samples were allowed to clot at room temperature for 2 h and centrifuged at 2500 r.p.m. for 15 min to obtain serum. Serum cholesterol was measured by the Immunochemical Laboratory Core Facility at the Mayo Clinic using an automated procedure (Roche Diagnostic System, Los Angeles, CA, USA). Cholesterol esters were hydrolyzed into cholesterol and free fatty acids by a cholesterol esterase. The cholesterol was then oxidized by cholesterol oxidase, producing hydrogen peroxide. The hydrogen peroxide was reacted with 4-amino-antipyrine and phenol, in the presence of peroxidase, to form a chromophore in an amount that was directly proportional to the cholesterol concentration and was read spectrophotometrically at 500 nm.

Histology of uterus

Five micron thick, paraffin embedded, hematoxylin- and eosin-stained uterine sections were used to determine epithelial cell height (μm) by light microscopy. The measurements reflected the mean height of the epithelial cell layer at numerous sites totaling a minimum perimeter length of 1·5 mm using Osteomeasure software.

Mammary gland sections and proliferation assay

PCNA was used to measure cells in S-phase as an index of proliferation. Five micron methacarn-fixed tissue sections were deparaffinized in xylene and rehydrated through a descending series of alcohol to water. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The primary antibody, PCNA (Dako, Carpenteria, CA, USA), was applied to the tissue at a dilution of 1:50 for 60 min. The secondary antibody, a biotinylated rabbit anti-mouse antibody, was diluted 1:200 in 10% normal rabbit serum and incubated for 30 min. Finally, streptavidin–HRP (Dako, Carpenteria, CA, USA) at a dilution of 1:1000 was applied to the sections and incubated for 30 min. The PCNA signal was visualized using 3,3′ diaminobenzidine. All washes were performed in modified PBS (0·04 M K_2HPO_4, 0·01 M NaH_2PO_4, 0·13 M NaCl). Sections were lightly counterstained in modified Harris hematoxylin (diluted 1:10), followed by an ascending series of alcohol and xylene, and mounted with Permount. Computer-generated random numbers provided coordinates for measurement of PCNA incorporation. Forty high-powered fields (40 ×) were identified for each animal. Cells that incorporated PCNA, as identified by brown pigment over the nuclei and along the nuclear membrane, were counted and expressed as a percentage of the total nuclear area measured using a Cell Systems Analysis System (Chicago, IL, USA).

Statistical analyses

All data are presented as means ± S.E.M. Unpaired *t*-test was used to determine the different between sham and OVX animals (study I). One-way ANOVA with Fisher's protected least significant difference *post-hoc* multiple comparison tests were used to compare the differences between multiple pairs of groups (studies II and III). Two-way ANOVA was performed to established the respective effects of E_2 and 2-OHE_1 or E_2 and 16α-OHE_1 (study II). A P value less than 0·05 was considered to be statistically significant.

Results

Study I

Twenty-seven days after surgery, body weight gain in OVX rats was significantly greater than that in sham controls (279 ± 3 compared with 232 ± 3 g). Ovariectomy had no effect on cancellous bone volume at the epiphysis, but increased dynamic indices of bone formation (double-labeled surface/bone surface, mineral apposition rate and bone formation rate expressed per bone surface (BS), bone volume (BV) or tissue volume (TV)) as shown in Table 1. It also resulted in the expected increase in longitudinal bone growth and decrease in uterine wet weight (data not shown).

Table 2 Effects of continuous release of 2-OHE_1, 16α-OHE_1, E_2+vehicle, and combinations of E_2 and 2-OHE_1, and E_2 and 16α-OHE_1 on body weight and serum cholesterol in OVX rats. Values are means ± S.E.M. in baseline (n=10), vehicle (n=8), 2-OHE_1 (n=9), 16α-OHE_1 (n=9), E_2+vehicle (n=9, or 6 as indicated), E_2+2-OHE_1 (n=11, or 9 as indicated) and E_2+16α-OHE_1 (n=9) groups

	Body weight		**Serum concentration**
	Initial (g)	Final (g)	Cholesterol (mg/dl)
Group			
Baseline	182 ± 3		92 ± 4
Vehicle	184 ± 3	288 ± 4[a]	102 ± 4[a]
2-OHE_1	186 ± 2	285 ± 6[a]	119 ± 3[ab]
16α-OHE_1	184 ± 3	283 ± 3[a]	112 ± 2[ab]
E_2+vehicle	182 ± 3	224 ± 3[abcd]	117 ± 2[ab] (6)
E_2+2-OHE_1	184 ± 2	224 ± 3[abcd]	134 ± 4[abcde] (9)
E_2+16α-OHE_1	184 ± 3	219 ± 3[abcd]	123 ± 4[abdf]
Two-way ANOVA			
(i) E_2	NS	0·0001	0·0002
2-OHE_1	NS	NS	0·0001
Interaction	NS	NS	NS
(ii) E_2	NS	0·0001	0·0005
16α-OHE_1	NS	NS	0·0181
Interaction	NS	NS	NS

P<0·05 compared with: [a]baseline group; [b]vehicle group; [c]2-OHE_1 group; [d]16α-OHE_1 group; [e]E_2+vehicle group; [f]E_2+2-OHE_1 group.

Study II

Table 2 shows the effects of 2-OHE_1, 16α-OHE_1, E_2, and combination of E_2 and 2-OHE_1, and E_2 and 16α-OHE_1 pellets on body weight, and serum cholesterol. All groups of animals gained weight during the 3-week treatment interval. Also, serum cholesterol increased in all groups compared with baseline. E_2 antagonized weight gain and increased serum cholesterol. Neither estrogen metabolite affected body weight. Both 2-OHE_1 and 16α-OHE_1 increased serum cholesterol. No interactions between E_2 and the estrogen metabolites were observed for body weight or serum cholesterol.

The cortical bone histomorphometry is summarized in Table 3. There was an age-related change due to radial bone growth as seen by increases in cross sectional and cortical bone area of all six treatment groups compared with the baseline group. No changes in static measurements were observed after one-way ANOVA among treatment groups, except for a reduced increase in cross sectional and marrow areas in rats treated with E_2+2-OHE_1. Two-way ANOVA revealed that E_2 rather than 2-OHE_1 exerted those effects. For dynamic measurements, E_2 and 16α-OHE_1 but not 2-OHE_1 resulted in decreases in mineral apposition rate and periosteal bone formation compared with vehicle. Two-way ANOVA indicated 16α-OHE_1 and E_2 decreased double-labeled perimeter in addition to mineral apposition rate and periosteal bone formation. There was no interaction between E_2 and the two metabolites for cortical bone measurements.

Longitudinal growth rate at the tibial metaphysis was significantly suppressed in animals treated with E_2 and 16α-OHE_1 but not 2-OHE_1 (Fig. 1). There was no interaction between E_2 and the metabolites, but E_2+16α-OHE_1 induced an additive effect.

The changes in cancellous histomorphometry are summarized in Table 4. Estrogen metabolites had no effect on

Table 3 Effects of continuous release of 2-OHE_1, 16α-OHE_1, E_2+vehicle, and combinations of E_2 and 2-OHE_1, and E_2 and 16α-OHE_1 on cortical bone in OVX rats. Values are means ± S.E.M.

	Cross section area (mm^2)	**Cortical bone area** (mm^2)	**Medullary area** (mm^2)	**Double-labeled perimeter** (mm)	**Mineral apposition rate** (μm/day)	**Periosteal bone formation** ($\times 10^{-3}$ mm^2/day)
Group						
Baseline (n=10)	4·00 ± 0·05	3·09 ± 0·04	0·91 ± 0·04			
Vehicle (n=8)	4·45 ± 0·07[a]	3·47 ± 0·05[a]	0·99 ± 0·04	7·51 ± 0·12	2·89 ± 0·12	21·81 ± 1·17
2-OHE_1 (n=9)	4·36 ± 0·08[a]	3·38 ± 0·04[a]	0·98 ± 0·06	6·98 ± 0·20	2·62 ± 0·19	18·37 ± 1·48
16α-OHE_1 (n=9)	4·37 ± 0·10[a]	3·41 ± 0·08[a]	0·97 ± 0·05	6·88 ± 0·17	2·33 ± 0·12[b]	16·18 ± 1·19[b]
E_2+vehicle (n=9)	4·28 ± 0·07[a]	3·38 ± 0·07[a]	0·91 ± 0·04	5·53 ± 0·34[bcd]	2·15 ± 0·10[bc]	11·96 ± 1·04[bcd]
E_2+2-OHE_1 (n=11)	4·21 ± 0·08[ab]	3·36 ± 0·05[a]	0·85 ± 0·04[bc]	5·38 ± 0·40[bcd]	2·11 ± 0·15[bc]	11·83 ± 1·65[bcd]
E_2+16α-OHE_1 (n=9)	4·28 ± 0·08[a]	3·34 ± 0·06[a]	0·94 ± 0·04	4·59 ± 0·44[bcde]	1·72 ± 0·11[bcdef]	8·06 ± 1·07[bcdef]
Two-way ANOVA						
(i) E_2	0·0407	NS	0·0349	0·0001	0·0002	0·0001
2-OHE_1	NS	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS	NS
(ii) E_2	NS	NS	NS	0·0001	0·0001	0·0001
16α-OHE_1	NS	NS	NS	0·0149	0·0001	0·0002
Interaction	NS	NS	NS	NS	NS	NS

P<0·05 compared with: [a]baseline group; [b]vehicle group; [c]2-OHE_1 group; [d]16α-OHE_1 group; [e]E_2+vehicle group; [f]E_2+2-OHE_1 group.

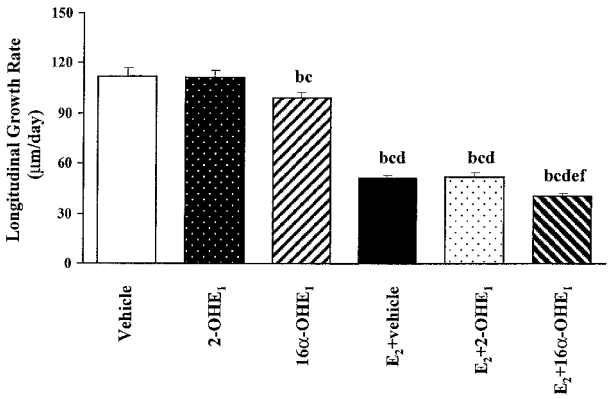

Figure 1 Tibial longitudinal growth rates in OVX rats receiving continuous release of vehicle, 2-OHE_1, 16α-OHE_1, E_2+vehicle, E_2+ 2-OHE_1 and E_2+ 16α-OHE_1. The values are means+S.E.M. Significant differences ($P<0·05$) compared with: [b]vehicle; [c]2-OHE_1; [d]16α-OHE_1; [e]E_2+vehicle; [f]E_2+2-OHE_1. Two-way ANOVA revealed significant effects of E_2 and 16α-OHE_1, no effect of 2-OHE_1 and no interaction between the estrogen metabolites and E_2.

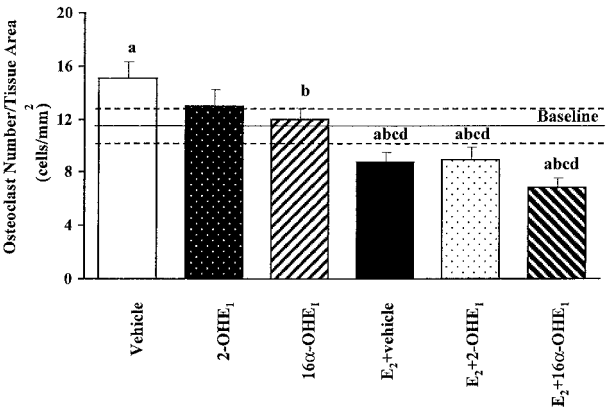

Figure 2 Osteoclast number/tissue area in OVX rats receiving continuous release of vehicle, 2-OHE_1, 16α-OHE_1 E_2+vehicle, E_2+ 2-OHE_1 and E_2+ 16α-OHE_1. The values are means+S.E.M. Significant differences ($P<0·05$) compared with: [a]baseline; [b]vehicle; [c]2-OHE_1; [d]16α-OHE_1. Two-way ANOVA revealed significant effects of E_2 and 16α-OHE_1, no effect of 2-OHE_1 and no interaction between the estrogen metabolites and E_2.

BV/TV. Compared with vehicle group, E_2 treatment resulted in decreases in double-labeled surface, mineral apposition rate and bone formation rate. In contrast to cortical bone, not only 16α-OHE_1 but also 2-OHE_1 decreased bone formation rate whether expressed as BS, BV or TV. Two-way ANOVA revealed that the effects of 16α-OHE_1 and E_2 were largely additive. The one exception was bone formation rate (BFR/BV), for which a significant interaction term was noted, indicating similar but non-additive actions. Likewise, the actions of 2-OHE_1 and E_2 on BFR/BV and BFR/TV were similar but non-additive. The inhibitory effect of 2-OHE_1 on BFR/BS noted by one-way ANOVA was not significant by two-way ANOVA.

The effects of E_2, 2-OHE_1 and 16α-OHE_1 pellets on osteoclast number are presented in Fig. 2. Compared with baseline, cancellous bone architecture measured at the epiphysis was unchanged in the vehicle-treated group (data not shown) but osteoclast number increased in the zone of vascular invasion. One-way and two-way ANOVA revealed decreases in osteoclast number in E_2- and 16α-OHE_1-treated animals, and no effect of 2-OHE_1.

Table 4 Histomorphometric measurement of cancellous bone in OVX rats receiving continuous release of vehicle, 2-OHE_1, 16α-OHE_1, E_2+vehicle, E_2+2-OHE_1, and E_2+16α-OHE_1. Values are means ± S.E.M.

	Bone volume/ tissue volume (%)	**Double-labeled surface/bone surface** (%)	**Mineral apposition rate** (μm/day)	**Bone formation rate** (BFR) BFR/BS (μm³/μm² per day)	BFR/BV (%/day)	BFR/TV (%/day)
Group						
Vehicle (n=8)	36·47 ± 1·58	23·02 ± 2·01	1·42 ± 0·09	0·33 ± 0·03	0·68 ± 0·08	0·24 ± 0·03
2-OHE_1 (n=9)	38·41 ± 1·28	19·53 ± 1·47	1·21 ± 0·05[b]	0·24 ± 0·02[b]	0·45 ± 0·05[b]	0·17 ± 0·01[b]
16α-OHE_1 (n=9)	40·15 ± 1·62	17·00 ± 1·18[b]	1·17 ± 0·04[b]	0·20 ± 0·02[b]	0·36 ± 0·04[b]	0·14 ± 0·02[b]
E_2+vehicle (n=9)	39·99 ± 1·38	13·53 ± 0·78[bc]	1·25 ± 0·05[b]	0·17 ± 0·01[bc]	0·28 ± 0·02[bc]	0·11 ± 0·01[bc]
E_2+2-OHE_1 (n=11)	38·56 ± 1·28	14·11 ± 1·81[bc]	1·11 ± 0·05[b]	0·16 ± 0·02[bc]	0·29 ± 0·04[bc]	0·11 ± 0·02[bc]
E_2+16α-OHE_1 (n=9)	37·70 ± 0·92	8·20 ± 0·70[bcdef]	1·02 ± 0·06[bce]	0·08 ± 0·01[bcdef]	0·15 ± 0·02[bcdef]	0·06 ± 0·01[bcdef]
Two-way ANOVA						
(i) E_2	NS	0·0001	0·0271	0·0001	0·0001	0·0001
2-OHE_1	NS	NS	0·0045	NS	0·0284	0·0331
Interaction	NS	NS	NS	NS	0·0168	0·0384
(ii) E_2	NS	0·0001	0·0128	0·0001	0·0001	0·0001
16α-OHE_1	NS	0·0001	0·0005	0·0001	0·0001	0·0001
Interaction	0·0398	NS	NS	NS	0·0234	NS

$P<0·05$ compared with: [b]vehicle group; [c]2-OHE_1 group; [d]16α-OHE_1 group; [e]E_2+vehicle group; [f]E_2+2-OHE_1 group.

Table 5 Effects of continuous release of 2-OHE_1, 16α-OHE_1, E_2+vehicle, and combinations of E_2 and 2-OHE_1, and E_2 and 16α-OHE_1 on uterine weight, epithelial cell height, and PCNA labeling. Values are means ± S.E.M. in baseline (n=10, or 9 as indicated), vehicle (n=8), 2-OHE_1 (n=9), 16α-OHE_1 (n=9), E_2+vehicle (n=9), E_2+2-OHE_1 (n=10, or 9 as indicated) and E_2+16α-OHE_1 group (n=9)

	Uterus		Mammary gland
	Weight (g)	Epithelial cell height (μm)	PCNA labeling (% cells)
Group			
Baseline	0·180 ± 0·005	14·54 ± 0·41 (9)	
Vehicle	0·103 ± 0·005[a]	12·20 ± 0·43	1·18 ± 0·30
2-OHE_1	0·106 ± 0·004[a]	14·69 ± 0·40	0·88 ± 0·36
16α-OHE_1	0·101 ± 0·003[a]	11·99 ± 0·30	1·10 ± 0·28
E_2+vehicle	0·408 ± 0·016[abcd]	37·14 ± 1·27[abcd]	5·77 ± 1·28[bcd]
E_2+2-OHE_1	0·363 ± 0·041[abcd]	33·92 ± 3·52[abcd]	7·00 ± 1·96[bcd] (9)
E_2+16α-OHE_1	0·416 ± 0·031[abcd]	32·09 ± 1·34[abcde]	6·01 ± 1·97[bcd]
Two-way ANOVA			
(i) E_2	0·0001	0·0001	0·0001
2-OHE_1	NS	NS	NS
Interaction	NS	NS	NS
(ii) E_2	0·0001	0·0001	0·0001
16α-OHE_1	NS	0·0119	NS
Interaction	NS	0·0199	NS

P<0·05 compared with: [a]baseline group; [b]vehicle group; [c]2-OHE_1 group; [d]16α-OHE_1 group; [e]E_2+vehicle group.

Uterine wet weight and epithelial cell height were measured (Table 5) to evaluate uterotropic estrogenic activity of the metabolite pellets. Compared with the baseline values obtained 6 days after ovariectomy, uterine wet weight in the vehicle group was decreased, but there was no change in epithelial cell height. Neither 2-OHE_1 nor 16α-OHE_1 had any additional effect on uterus. In contrast, E_2 increased both uterine wet weight and epithelial cell height. 2-OHE_1 did not have any effect on E_2-induced uterine hypertrophy. Interestingly, E_2+16α-OHE_1 decreased epithelial cell height, with no change in uterine wet weight. This antagonistic effect of 16α-OHE_1 on E_2-stimulated increase in epithelial cell height was supported by a significant interaction of E_2 and 16α-OHE_1 by two-way ANOVA.

Mammary gland data are shown in Table 5. 2-OHE_1 and 16α-OHE_1 did not change PCNA labeling compared with that in vehicle-treated animals, but PCNA labeling was increased in the mammary glands excised from animals receiving E_2 or combinations of E_2 and the metabolites.

Study III

Figure 3 shows the dose–response effects of daily s.c. administration of 16α-OHE_1 on uterine wet weight. Similar to the effects in mature OVX rats, E_2 exhibited a pronounced uterotropic effect in weanling rats. In contrast to continuous administration with pellets, daily injection of 16α-OHE_1 at a dose rate as low as 300 μg/kg per day increased uterine wet weight, and the uterine weight

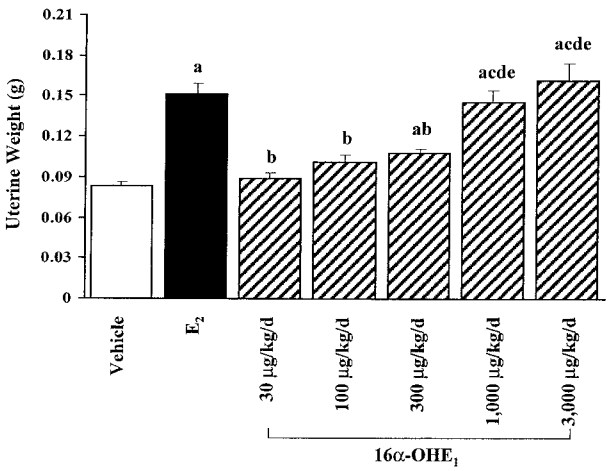

Figure 3 Uterine weight in weanling rats receiving daily injection of vehicle, E_2, and 30, 100, 300, 1000 and 3000 μg/kg per day (d) 16α-OHE_1. The values are means+S.E.M. Significant differences (P<0·05) compared with: [a]vehicle; [b]E_2; [c]30 μg/kg per day 16α-OHE_1; [d]100 μg/kg per day 16α-OHE_1; [e]300 μg/kg per day 16α-OHE_1.

induced by the 1000 and 3000 μg/kg per day dose rates was similar to that in E_2-treated group.

Discussion

The present investigation highlights the differential biological responses of bone and reproductive tissues to continuous exposure to E_2, 2-OHE_1 and 16α-OHE_1. This experiment was also designed to detect interactions between E_2 and the estrogen metabolites by choosing a dose of E_2 known to elicit submaximal estrogenic activity on target tissues (Sibonga *et al.* 1998). The results demonstrated that 16α-OHE_1 was an estrogen agonist with respect to serum cholesterol, longitudinal bone growth and cortical and cancellous bone measurements and a weak estrogen antagonist with respect to E_2-induced uterine hypertrophy, whereas 2-OHE_1 was an estrogen agonist with respect to serum cholesterol and selected indices of cancellous bone turnover and without effect on reproductive tissues.

The estrogen agonistic effects elicited by either 2-OHE_1 or 16α-OHE_1 in this study appeared not to be the result of their biotransformation to E_2, because serum concentrations of E_2 in 2-OHE_1- and 16α-OHE_1-treated animals were similar to those in the vehicle-treated group and less than that in E_2-treated group (data not shown). We cannot rule out that some of the observed negative effects resulted from tissue specific *O*-methylation of 2-OHE_1 to 2-methoxyestrone. This estrogen metabolite has a much reduced affinity for estrogen receptors and is likely to be inactive. Similarly, at peripheral sites 16α-OHE_1 can be metabolized to estriol, which has a greater affinity for sex hormone binding globulin.

Previous studies using s.c. injection showed no effect of 2-OHE_1 on any of the estrogen target tissues evaluated (Westerlind *et al.* 1998). 2-OHE_1, which is known to bind to the classical estrogen receptor with a markedly reduced binding affinity compared with that of the parent hormone (Ball & Knuppen 1980, MacLusky *et al.* 1983, Van Aswegen *et al.* 1989, Feigelson & Henderson 1996), is unlikely to cause the long-term receptor occupancy in the nucleus of target cells necessary to produce estrogenic activity after brief exposure. In contrast, continuous infusion has been shown to have effects on some estrogen target tissues (Fishman & Martucci 1980). This is, however, the first study showing any activity of 2-OHE_1 on either the skeleton or serum cholesterol. We cannot rule out the possibility that greater dose rates of 2-OHE_1 would have had greater activity. However, the previous study (Westerlind *et al.* 1998) resulted in greatly increased concentrations of the metabolite and larger amounts were given in the present study. Thus it is unlikely that physiological circulating levels of 2-OHE_1 have activity on bone and other E_2 target tissues.

Ovariectomy has been reported to increase serum cholesterol and this change can be prevented by treatment with either E_2 (Black *et al.* 1994) or 16α-OHE_1 (Westerlind *et al.* 1998). E_2 was reported to decrease serum cholesterol by up-regulation of hepatic low-density lipoprotein receptor (Staels *et al.* 1989). The failure to decrease serum cholesterol with E_2 and its metabolites observed in this study is not unique. Earlier findings of normocholesterolemia or mild hypercholesterolemia have been reported in animals receiving E_2 (Ke *et al.* 1995, Westerlind *et al.* 1998). The effect of E_2 may depend in part upon the method of hormone administration: subcutaneous administration of E_2 results in hypercholesterolemia, whereas oral administration results in hypocholesterolemia, suggesting that first-pass metabolism in the liver is essential for cholesterol decreasing. This conclusion is supported by the failure to detect cholesterol decreasing by E_2 in some transdermal studies in women (Hanggi *et al.* 1993).

The tibial shaft and proximal epiphysis are estrogen target sites (Westerlind *et al.* 1997, 1998). Estrogen deficiency induced by ovariectomy results in an increase in periosteal bone growth in the shaft, in addition to increased bone turnover at the epiphysis. The magnitude of change in cancellous bone turnover at the epiphysis is comparable to or greater than that observed at the more commonly measured metaphysis (Wronski *et al.* 1985, Turner *et al.* 1987, Westerlind *et al.* 1994). Dynamic bone measurements in the epiphysis, unlike those in the metaphysis, are not influenced by growth in the young sexually mature rat. In addition, even prolonged estrogen deficiency has no effect on cancellous bone volume of the epiphysis, whereas the metaphysis becomes severely osteopenic (Westerlind *et al.* 1997). Our data demonstrated that E_2 decreased periosteal bone formation rate by 45%, whereas it reduced cancellous bone formation rate by 48%, 59% and 54%, when normalized to BS, BV and TV respectively. Similar results were obtained in 16α-OHE_1-treated ovariectomized rats. Thus, 16α-OHE_1 influenced both bone growth and bone turnover. 2-OHE_1, in contrast, had limited effects on cancellous bone.

The basis for any responses to estrogen metabolites is believed to be mediated by their direct interaction with E_2 receptor. The importance of the E_2 receptor is supported by cell culture studies using the human fetal osteoblastic cell line, hFOB/ER9, in which the effects of 16α-OHE_1 were abolished by the estrogen receptor antagonist ICI 182780 (Robinson *et al.* 2000).

Estrogen is required for proliferation and differentiation of mammary tissue. 16α-OHE_1 binds to E_2 receptor and has been implicated in developing mammary tumors (Schneider *et al.* 1982, Bradlow *et al.* 1985, 1986). However, the lack of 16α-OHE_1 effect on PCNA labeling in this study is similar to a previous finding (Westerlind *et al.* 1998). Furthermore, more recent studies question the role of 16α-hydroxylation of estrogen in tumor development (Gupta *et al.* 1998).

It was reported that 2-OHE_1 exhibited partial estrogen receptor antagonism in MCF-7 breast cancer cells, as a result of the production of free radicals during metabolic redox cycling, to induce DNA damage (Schneider *et al.* 1984, Vandewalle & Lefebvre 1989). However, we observed no effect of continuous release 2-OHE_1 pellets on mammary gland. Stimulation may not occur in mammary tissue *in vivo* because the product of local *O*-methylation of the 2-hydroxylated estrogen metabolite undergoes a rapid clearance (Lipsett *et al.* 1983, Emons *et al.* 1987).

Uterine wet weight in the vehicle group was less than that of the baseline group. In contrast, the epithelial cell height did not differ between the two groups, suggesting that some but not all of the effects of OVX on the uterus were fully established at the start of the experiment. Uterine epithelial cell height in age-matched ovary-intact rats from a parallel study was 19·32 ± 1·07 μm, confirming that OVX resulted in a decrease in the thickness of the epithelium (unpublished data). Continuous infusion of 2-OHE_1 did not increase uterine wet weight or increase epithelial cell height when administered to OVX animals, nor did it antagonize the stimulatory actions of estrogen. Although 2-OHE_1 has been shown to act as a weak estrogen antagonist in some cell culture models (Schneider *et al.* 1984, Vandewalle & Lefebvre 1989), our results demonstrate that this estrogen metabolite is unlikely to function as an anti-estrogen in reproductive tissues *in vivo*.

The observed dose–response effects of daily s.c. administration of 16α-OHE_1 on uterine weight in weanling rats confirmed previous findings in 3-month-old rats (Westerlind *et al.* 1998). These findings contrast with those from continuous infusion of 16α-OHE_1 which was not an estrogen agonist with respect to either uterine wet weight or epithelial cell height and was a weak estrogen antagonist with respect to epithelial cell height. Thus the tissue-selective actions of this estrogen metabolite are most pronounced when it is continuously present in the circulation.

SERMs, such as tamoxifen and raloxifene, exhibit tissue-selective estrogen agonism which is superficially similar to that of 16α-OHE_1. However, the respective mechanisms of action of these two classes of estrogen appear to differ. SERMs compete effectively with E_2 for ligand binding sites on estrogen receptors. As a consequence, the tissue-selective agonistic/antagonistic profile of SERMs is inherent to how these compounds bind to the ligand binding site (Evans & Turner 1995). In contrast, neither estrogen metabolite was very effective in antagonizing the stimulatory actions of a submaximal dose of E_2 on reproductive tissues. This observation, combined with their inactivity in the reproductive tract in the absence of E_2, strongly suggests that the tissue-selective actions of estrogen metabolites are related to local metabolism.

Uterine growth requires retention of the estrogen receptor–ligand complex in the nucleus of target cells (Anderson *et al.* 1975). 16α-OHE_1 and 2-OHE_1 have much lower affinities for the estrogen receptor than E_2 and thus must be present at greater concentrations to be effective. A greater rate of clearance in reproductive tissues compared with that in bone could contribute to the observed tissue selective actions of the metabolites. This speculation is supported by the observation that the method of delivery of 16α-OHE_1 has a dramatic influence upon the uterotropic response to 16α-OHE_1.

In summary, our data confirm that two important estrogen metabolites in postmenopausal women, 2-OHE_1 and 16α-OHE_1, have distinctly different activities on bone metabolism in growing female rats. 16α-OHE_1 is an estrogen agonist, whereas 2-OHE_1 had minimal activity. These findings may be relevant to determining individual differences in the rates of bone loss after the menopause. In addition, the estrogen metabolites exhibited target-tissue specificity, which was more probably due to differences in their local metabolism than to SERM-like activity. This latter conclusion, if confirmed, reveals a novel strategy for optimizing hormone replacement therapy.

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