The catechol estrogen, 4-hydroxyestrone, has tissue-specific estrogen actions

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Abstract

Recent data indicate that the catechol estrogen, 2-hydroxyestrone (2-OHE_1), has no effect on any target tissue including bone, whereas 16α-hydroxyestrone (16α-OHE_1) exerts tissue-selective estrogen agonist activity. The effect of the catechol estrogen, 4-hydroxyestrone (4-OHE_1), putatively associated with tumorigenesis, has not been studied in the skeleton. The purpose of this study was to assess the effect of 4-OHE_1 on tibia, uterine and mammary gland histology and blood cholesterol in ovariectomized (OVX'd) growing rats. Ten-week-old female Sprague–Dawley rats were injected subcutaneously with 200 μg/kg BW per day with 4-OHE_1, 17β-estradiol (E_2) or vehicle for three weeks. OVX resulted in uterine atrophy, increased body weight, radial bone growth and cancellous bone turnover, and hypercholesterolemia. E_2 prevented these changes with the expected exception that the subcutaneous infusion of this high dose of estrogen did not prevent the hypercholesterolemia. 4-OHE_1 prevented the increase in blood cholesterol and the increase in body weight. 4-OHE_1 appeared to have partial estrogen activity in the uterus; uterine weight and epithelial cell height were significantly greater than the OVX rats but significantly less (twofold) than the E_2 animals. Analysis of variance indicated that 4-OHE_1 slightly decreased the periosteal mineral apposition rate ($P<0{\cdot}05$) compared with vehicle-treated rats but had no effect on double-labeled perimeter or bone formation rate. Similarly, 4-OHE_1 was a partial estrogen agonist on cancellous bone turnover. The data suggest that the catechol estrogen, 4-OHE_1, unlike 2-OHE_1, has estrogen activity. Furthermore, the profile of activity differs from that of 16α-OHE_1. Our results suggest that estrogen metabolites may selectively influence estrogen-target tissues and, concomitantly, modulate estrogen-associated disease risk.

Journal of Endocrinology (2000) **167,** 281–287

Introduction

The presence or absence of estrogens are implicated in the etiology and pathogenesis of multiple diseases including breast and reproductive cancers, osteoporosis, and coronary heart disease. Current investigations are underway to evaluate their role in Alzheimer's, stroke, and cancers of the colon and prostate.

Early evidence has suggested that breakdown of estradiol and estrone to their metabolites (2,4,16α-hydroxyestradiol and 2,4,16α-hydroxyestrone) may be indicative of risk of disease. For example, it has been observed that increased 2-hydroxylation of estrone to the catechol estrogen, 2-hydroxyestrone (2-OHE_1), confers decreased risk for breast (Ho *et al.* 1998, Meilahn *et al.* 1998) and cervical cancer (Sepkovic *et al.* 1995) but may be associated with an increased risk for osteoporosis (Hodge *et al.* 1995, Lim *et al.* 1997). Conversely, elevated 16α-hydroxylation to 16α-hydroxyestrone (16α-OHE_1) has been associated with increased risk for breast cancer (Schneider *et al.* 1982, Osborne *et al.* 1993, Kabat *et al.* 1997) but decreased risk for osteoporosis (Lim *et al.* 1997). Although these hypotheses are not proven, it is evident that the estrogen metabolites are not breakdown products without biological activity but rather have specific and differential activities in estrogen-sensitive tissues.

In this vein, this lab recently evaluated the effect of 2- and 16α-OHE_1 on estrogen-responsive tissues including the uterus, liver, mammary gland and bone in the ovariectomized rat (Westerlind *et al.* 1998). The catechol estrogen, 2-OHE_1, had no estrogen activity on any target tissue evaluated. In contrast, 16α-OHE_1 acted as a full estrogen agonist on the bone and liver and a partial estrogen agonist on the uterus and mammary gland. In this regard, this endogenous estrogen resulted in similar effects to those seen with anti-estrogens such as tamoxifen and raloxifene (Evans *et al.* 1994, Ke *et al.* 1995, Evans *et al.* 1996, Turner *et al.* 1988).

Although not the predominant pathway in humans, estradiol and estrone are also hydroxylated at the C-4

0022–0795/00/0167–281

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position to form the catechol estrogens, 4-hydroxyestradiol (4-OHE_2) and 4-hydroxyestrone (4-OHE_1) respectively (Ball & Knuppen 1980). These metabolites have been shown to be tumorigenic in animal models (Liehr 1994, Yager & Liehr 1996) as they undergo metabolic redox cycling to generate free radicals and unstable adducts capable of removing purines from DNA and potentiating the misrepair of important genes. Thus, although it is generally accepted that certain estrogen metabolites increase cell proliferation and are associated with cancer promotion and progression, it is also speculated that some estrogen metabolites may be associated with cancer initiation.

To date, the effect of 4-OHE_1 on other estrogen-sensitive tissues has not been thoroughly evaluated. The purpose of the present investigation was to characterize the response of the uterus, mammary gland, liver and bone to 4-OHE_1 treatment in the ovariectomized rat model. This study would complement those data from earlier work and would extend our understanding of the relative estrogenicity of three important estrogen metabolites in multiple tissues.

Materials and Methods

Fifty female Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY, USA) at approximately 9 weeks of age (mean BW = 170 ± 2 g). Bilateral ovariectomy (OVX) (n = 40) or sham surgery (n = 10; Intact) was performed at the vendor 2–3 days prior to shipment. Upon arrival and following acclimation to the facilities, the 40 OVX animals were stratified by weight and randomly assigned to four groups (n = 10 for each group), consisting of a baseline or one of three treatment groups: 4-OHE_1, 17β-estradiol (E_2) or vehicle (OVX).

Treatment was not started until 1 week post-OVX to allow for estrogen levels to become depleted. Treatment consisted of daily subcutaneous injections of 4-OHE_1, 17β-E_2 or vehicle (50% ethanol). The dose was set at 200 μg/kg BW based on an initial mean group body weight of 180 g and was not altered over the 3-week treatment period. This dose was chosen because it had been used in the previous study with 2- and 16-OHE_1 and would ensure saturation of estrogen receptors. 4-OHE_1 and 17β-E_2 were obtained from Sigma Chemical Co. (St Louis, MO, USA) and mixed to appropriate concentrations in the vehicle. Ascorbic acid (1 mg/ml) was added to the preparation of 4-OHE_1 to prevent oxidation of the labile metabolite. No ascorbic acid was added to the vehicle or E_2 solution. To further maintain biological activity, solutions were aliquoted to individual vials for daily injections and frozen at −80 °C until use. All animals were injected with 200 μl of the individual treatment at 08:00 daily. Treatment duration was 3 weeks.

Fluorochromes to label mineralizing bone matrix were administered by juxta-tail vein injection 1 day before starting the treatment period (tetracycline-HCl, 20 mg/kg of BW; Sigma), 7 days before sacrifice (calcein, 20 mg/kg of BW; Sigma), and 1 day before sacrifice (tetracycline, as described). The two fluorochome labels are readily differentiated under u.v. illumination, because tetracycline fluoresces pale yellow, while calcein fluoresces bright green. Animals were injected i.p. with bromodeoxyuridine, in 0·9% NaCl ((BRDU); 50 mg/kg BW) 3 h prior to sacrifice to label cells in S-phase.

Animals were sacrificed by CO_2 inhalation 24 h after their last treatment injection. Baseline animals were sacrificed on the day that treatment was started. Blood was collected via the peri-orbital route for measurement of serum cholesterol. The uterus was excised for wet weight determination and for measurement of epithelial cell height. Mammary glands were excised for histological evaluation and BRDU immunohistochemistry quantitation. The right tibia was removed and fixed in 70% ethanol for static and dynamic histomorphometry.

Uterus

The uterus was weighed for an initial wet weight and then fixed in 10% neutral buffered formalin for 4–6 h, then transferred to 70% ethanol until processing for conventional paraffin embedding. Sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) to measure epithelial height. Uterine epithelial height, expressed in μm, was measured at × 40 with a Cell Analysis System (Chicago, IL, USA). A minimum of 50 sites were measured from each section/animal.

Mammary glands

The right and left mammary gland chains were removed. Lymph node regions were removed and fixed in 10% neutral buffered formalin and Methacarn. These regions were used for histological evaluation with standard H&E staining and BRDU immunohistochemistry respectively.

Mammary glands fixed in Methacarn were embedded in paraffin and sectioned at 5 μm. Sections were deparaffinized in a series of xylenes, hydrated through descending grades of ethanol, rinsed in deionized water and denatured in 2 N HCl for 90 min. After rinsing, sections were placed in 0·1 M sodium tetraborate for 5 min, then rinsed again in deionized water. The endogenous peroxidase activity was blocked with 3% H_2O_2 and followed with another rinse in PBS. The primary antibody, Mouse anti-BrdU (1:40) (Becton–Dickinson, San Diego, CA, USA) was applied followed by three rinses in PBS. Sections were incubated for 30 min with biotinylated Rabbit anti-Mouse (1:200) (Dako, Carpinteria, CA, USA) in 10% Normal Rabbit Serum. Sections were then incubated with conjugated HRP streptavadin (1:1000) (Dako) then rinsed in PBS. Diaminobenzidine (DAB) (Sigma) in 0·4% H_2O_2 was applied for 10 min and sections were rinsed in deionized

water and counterstained with Harris Hematoxylin and blued with Scotts H_2O. Sections were dehydrated through ascending grades of ethanol, cleared in xylene, and mounted with Permount (Fisher, Pittsburgh, PA, USA).

Computer-generated random numbers provided coordinates for measurement of BRDU incorporation. Twenty high powered fields were identified each for ductal and lobular structures. Cells that incorporated BRDU, as identified by brown pigment over the nuclei and along the nuclear membrane, were counted and expressed as a percentage of the area measured. The reviewer was blinded to group assignment.

Serum cholesterol

Blood samples were allowed to clot at room temperature for 2 h and serum was obtained after centrifugation at 2000 r.p.m. for 15 min. Serum samples were stored at −80 °C until analysis. Serum cholesterol was determined using a Cobas–Mira high performance cholesterol assay (Branchburg, NJ, USA) with Roche Reagents (Mannheim, Germany). Briefly, serum cholesterol is released from its esters by enzymatic action of an ester hydrolase and then oxidized by cholesterol oxidase to produce hydrogen peroxide. The hydrogen peroxide, when combined with 4-aminoantipyrine and phenol, forms a chromophore which is visible at 500 nm and is directly proportional to the cholesterol concentration.

Bone histomorphometry

Histomorphometric measurements were performed with an Osteomeasure semi-automatic image analysis system (Osteomeasure, Atlanta, GA, USA) which has been described in detail (Jiminez *et al.* 1997).

Longitudinal growth rate

Longitudinal growth rate was measured in the proximal tibial metaphysis and is the mean distance between the calcein labeling front located in the primary and secondary spongiosa and the final tetracycline label in the mineralizing growth plate cartilage divided by the labeling interval of 6 days.

Cortical bone measurements

Ground transverse sections were used for histomorphometric analysis of cortical bone. Cross-sections 150 μm thick were cut at a site just proximal to the tibia–fibula synostosis with a low speed saw (Isomet, Buehler, Lake Bluff, IL, USA) equipped with a diamond wafer blade. The sections were ground to a thickness of 20 μm on a roughened glass plate and mounted in glycerin before microscopic examination under u.v. illumination to visualize fluorochrome labeling. The following measurements were performed on the undemineralized sections as described (Jiminez *et al.* 1997): (1) cross-sectional area, defined as the area of bone and marrow cavity bounded by the periosteal surface of the specimen, (2) medullary area, defined as the area delineated by the endocortical surface of the specimen, (3) cortical bone area, calculated as the difference between the cross-sectional and medullary area, (4) periosteal perimeter, defined as the total perimeter enclosing the cross-section (periosteal perimeter includes fluorochrome-labeled and nonlabeled perimeters), (5) periosteal bone formation rate, calculated as the area bounded by the tetracycline labels and divided by the labeling period of 21 days, and (6) periosteal mineral apposition rate, defined as the periosteal bone formation rate divided by the label perimeter.

Cancellous bone measurements

The tibia was dehydrated in a series of increasing concentrations of ethanol, embedded without demineralization in a mixture of methylmethacrylate-2-hydroxyethylmethacrylate (12·5:1) to retain the fluorochrome labels, and sectioned at a thickness of 5 μm.

The sampling site included the entire tibial epiphysis and represented an area approximately 2·8 mm^2. Measurements were performed as described previously (Jiminez *et al.* 1997). Cancellous bone area was determined as the area of total cancellous bone per mm^2 epiphyseal area and expressed as a percentage. Cancellous bone perimeter was determined as the perimeter of cancellous bone perimeter per mm^2 epiphyseal area. Bone formation rate was calculated as the product of the double label perimeter and mineral apposition rate. The mineral apposition rate, expressed in μm/day was the mean distance between the calcein and second tetracycline label divided by the labeling interval of 6 days. Double-labeled perimeter was determined as the bone perimeter with the calcein and second tetracycline labels. The data were expressed in three-dimensional units following multiplication by unit thickness.

Statistical analysis

Analyses of variance were performed on all variables. Student–Neuman–Keuls *post hoc* multiple comparison tests were performed to assess between-group differences when appropriate. A P value of $<0{\cdot}05$ was designated as statistically significant.

Results

The effects of OVX, E_2 and 4-OHE_1 on body weight, serum cholesterol, and longitudinal growth rate are presented in Table 1. All of the treatment groups had an increase in body weight compared with baseline values.

Table 1 Effects of OVX, 17β-estradiol and 4-OHE_1 on body weight, serum cholesterol and longitudinal growth rate at the proximal tibial metaphysis. Values are means ± S.E.M.

	Baseline	Intact	OVX–vehicle	17β-estradiol	4-OHE_1
Measurement					
Initial weight (g)	170·7 ± 2·3	170·3 ± 2·9	170·8 ± 2·0	170·3 ± 2·7	170·0 ± 1·8
Final weight (g)		212·3 ± 4·0*	250·3 ± 6·5†	200·7 ± 4·2*	217·4 ± 4·1*
Growth rate (g/day)		2·1 ± 0·2*	4·0 ± 0·3†	1·5 ± 0·2*†	2·4 ± 0·2*
Cholesterol (mg/dl)		92·4 ± 4·8*	111·7 ± 4·7†	129·3 ± 5·9*†	98·6 ± 6·2*
Longitudinal growth rate (μm/day)		76 ± 7*	142 ± 10†	60 ± 8*	101 ± 3*†

*Significantly different ($P \leq 0{\cdot}05$) from the OVX–vehicle group.
†Significantly different ($P \leq 0{\cdot}05$) from the intact group.

OVX resulted in significant increases in body weight, serum cholesterol and longitudinal growth rate relative to the intact animals. E_2 prevented the OVX-induced weight gain and increase in longitudinal growth rate and resulted in values not different from the intact group. Serum cholesterol was elevated by treatment with E_2 to levels significantly greater than the OVX and intact animals. Treatment with 4-OHE_1 halted the OVX-induced body weight gain and serum cholesterol increase; values were not significantly different from the intact group. The increase in longitudinal growth rate was attenuated in the 4-OHE_1 treated animals to a level intermediate between the OVX and intact animals.

Uterine wet weight and epithelial height data are presented in Table 2. OVX resulted in significant decreases in wet weight and epithelial height relative to the intact group. E_2 treatment resulted in uterine weights and epithelial height significantly greater than the OVX and intact animals. 4-OHE_1 treatment resulted in uterine wet weights that were significantly greater than the OVX group but significantly less than those of the intact animals. Also, epithelial heights were significantly greater than OVX but not different from those of the intact animals. All 4-OHE_1 uterine values were significantly less than the E_2 treatment (not signified in Table 2).

BRDU labeling of lobular and ductal units from the lymph node region of the mammary gland (Table 2) indicated a significant increase as a result of E_2 treatment relative to OVX. No increase was observed with 4-OHE_1 relative to the OVX group. Data from the intact animals were not included due to the variability in labeling associated with the normal estrous cycle.

Cortical bone histomorphometry data are shown in Table 3. As expected in a short-term study, there were no significant differences in static bone measurements (cross-sectional area, medullary area, cortical bone area, periosteal perimeter) among the intact, OVX or two estrogen-treated groups. There was an age-related change in the four groups relative to the baseline animals in the cross-sectional and cortical areas and in periosteal perimeter expansion. OVX resulted in an increase in periosteal bone formation rate and mineral apposition rate that was prevented by E_2 treatment. 4-OHE_1 did not result in a decrease in bone formation rate relative to the OVX although the mineral apposition rate was attenuated to a level intermediate between the OVX and intact animals.

The high rate of longitudinal growth precluded measurement of cancellous bone remodeling in the tibial metaphysis. The epiphysis, in contrast, is skeletally mature bone and is thus amenable to studying bone remodeling in the young animal model. Although OVX does not generally result in a loss of cancellous bone from the epiphysis, bone turnover is greatly increased at that site. Cancellous bone histomorphometry data are presented in Table 4. OVX resulted in a significant increase in bone formation rate, whether expressed relative to tissue

Table 2 Effects of OVX, 17β-estradiol and 4-OHE_1 on uterine weight, uterine epithelial height and BRDU labeling of mammary gland. Values are means ± S.E.M.

	Intact	OVX–vehicle	17β-estradiol	4-OHE_1
Measurement				
Uterus				
Uterine wet weight (g)	0·38 ± 0·02*	0·10 ± 0·05†	0·44 ± 0·02*†	0·27 ± 0·01*†
Epithelial height (μm)	15·5 ± 0·8	10·4 ± 0·6†	29·9 ± 2·4*†	16·7 ± 1·1*
Mammary gland – BRDU labeling				
Ducts (% area)		1·24 ± 0·82	3·14 ± 0·90*	1·59 ± 0·47
Lobules (% area)		0·90 ± 0·46	3·50 ± 0·92*	1·14 ± 0·38

*Significantly different ($P \leq 0{\cdot}05$) from the OVX–vehicle group.
†Significantly different ($P \leq 0{\cdot}05$) from the intact group.

Table 3 Effects of OVX, 17β-estradiol and 4-OHE_1 on cortical bone histomorphometry. Values are means ± S.E.M.

	Baseline	Intact	OVX–vehicle	17β-estradiol	4-OHE_1
Measurement					
Cross-sectional area (mm^2)	3·21 ± 0·04	3·65 ± 0·06*	3·71 ± 0·07*	3·55 ± 0·07*	3·70 ± 0·10*
Medullary area (mm^2)	0·77 ± 0·03	0·70 ± 0·03	0·70 ± 0·03	0·60 ± 0·02	0·79 ± 0·05
Cortical bone area (mm^2)	2·44 ± 0·04	2·95 ± 0·05*	3·02 ± 0·05*	2·92 ± 0·05*	2·91 ± 0·07*
Periosteal perimeter (mm)	6·05 ± 0.05	6·97 ± 0·07*	7·07 ± 0·07*	6·80 ± 0·06*†	7·01 ± 0·09*
Periosteal bone formation rate ($mm^2 \times 10^{-3}$/day)		16·62 ± 0·99†	25·92 ± 1·51‡	19·01 ± 1·51†	23·76 ± 1·23‡
Mineral apposition rate (μm/day)		2·72 ± 0·11†	3·77 ± 0·21‡	2·91 ± 0·15†	3·32 ± 0·14†‡

*Significantly different ($P \leq 0·05$) from the baseline group.
†Significantly different ($P \leq 0·05$) from the OVX–vehicle group.
‡Significantly different ($P \leq 0·05$) from the intact group.

volume, bone volume or bone surface. E_2 treatment prevented the increases in the three indices of bone formation. 4-OHE_1 mitigated the OVX-induced increase in the indices of formation but not as greatly as E_2 treatment. Bone volume was increased in both the E_2 and 4-OHE_1-treated groups, in all likelihood due to an inhibition of bone resorption. The mineral apposition rate was not significantly altered by OVX or any treatment. The surface covered by double labels was significantly increased with ovariectomy, was prevented in full by E_2 treatment, and was partially prevented with 4-OHE_1.

Discussion

Ovariectomy resulted in the expected uterine and mammary gland atrophy while increasing body weight, serum cholesterol and indices of bone turnover (Wronski *et al.* 1986, 1988, Turner *et al.* 1987, Westerlind *et al.* 1993). E_2 treatment resulted in increases in uterine weight, mammary gland proliferation and serum cholesterol as has previously been documented (Westerlind *et al.* 1998). Likewise, E_2 treatment arrested the OVX-induced acceleration in both longitudinal bone growth, radial bone growth and cancellous bone turnover (Westerlind *et al.* 1993, 1998). Serum cholesterol was increased in the E_2-treated animals, a finding similar to that observed previously (Westerlind *et al.* 1998). Although not noted in human estrogen replacement studies, increases in cholesterol, due to dose and/or mode of administration, have been reported by other investigators in the rat model (Ke *et al.* 1995, Evans *et al.* 1996).

The catechol estrogen 4-OHE_1 had effects that were tissue specific. Serum cholesterol was markedly decreased in the 4-OHE_1 group and was not different from the intact animals. 4-OHE_1 was not as effective as E_2 in stimulating uterine growth. Values were greater than the OVX animals and approximately 40% of those of the E_2 animals. This is consistent with earlier reports of the relative estrogen activity of the 4-hydroxyestrogens in reproductive tissues (Martucci 1983).

Qualitative mammary gland histology indicated significant lobular/alveolar development in the 4-OHE_1 group compared with the OVX animals. In several cases, the glands were indistinguishable from those found in the E_2 group. This apparent increased proliferation is not however reflected in the BRDU quantitative immunohistochemistry. Animals had been treated for 3 weeks with

Table 4 Effects of OVX, 17β-estradiol and 4-OHE_1 on cancellous bone histomorphometry in the tibial epiphysis. Values are means ± S.E.M.

	Intact	OVX–vehicle	17β-estradiol	4-OHE_1
Measurement				
Bone volume/tissue volume (%)	34·0 ± 2·3	32·4 ± 3·0	43·3 ± 2·8*†	38·5 ± 2·4
Bone surface/tissue volume ($mm^2/mm^2 \times 10^{-3}$/day)	6·2 ± 0·3	6·3 ± 0·6	6·9 ± 0·4	6·2 ± 0·4
Bone formation rate/tissue volume (%/day)	0·09 ± 0·02*	0·33 ± 0·03†	0·11 ± 0·03*	0·20 ± 0·02*†
Bone formation rate/bone volume (%/day)	0·28 ± 0·06*	0·87 ± 0·14†	0·27 ± 0·08*	0·52 ± 0·05*†
Bone formation rate/bone surface ($\mu m^3/\mu m^2$ per day)	0·15 ± 0·03*	0·45 ± 0·07†	0·17 ± 0·05*	0·32 ± 0·04*†
Mineral apposition rate (μm/day)	1·6 ± 0·1	1·9 ± 0·1	1·8 ± 0·1	1·7 ± 0·1
Double-labeled surface/bone surface (%)	9·5 ± 1·6*	27·4 ± 1·4†	8·6 ± 2·0*	18·1 ± 2·1*†

*Significantly different ($P \leq 0·05$) from the OVX–vehicle group.
†Significantly different ($P \leq 0·05$) from the intact group.

Table 5 Relative activity of 2-OHE_1, 4-OHE_1 and 16α-OHE_1 as compared with 17β-estradiol. All values are percentages. Estrogen activity of 17β-estradiol treatment in the OVX rat was set at 100%. The effects of the metabolites are expressed as a percentage of those observed for the 17β-estradiol treated groups relative to the OVX–vehicle group. Data for 2- and 16α-OHE_1 were obtained previously (Westerlind *et al.* 1998)

	2-OHE_1	4-OHE_1	16α-OHE_1	17β-estradiol
Measurement				
Body weight change	18	66	63	100
Uterus				
Wet weight	0	50	36	100
Epithelial height	0	32	26	100
Mammary gland proliferation*	11	13	33	100
Longitudinal bone growth	18	50	77	100
Cortical bone				
Bone formation rate	−24	31	80	100
Mineral apposition rate	−21	52	65	100
Cancellous bone				
Bone formation rate/bone surface	23	46	75	100
Double-labeled surface/bone surface	18	49	77	100

*Proliferation was measured by PCNA (2-OHE_1, 16α-OHE_1) and BRDU (4-OHE_1) labeling.

4-OHE_1 and it could be expected that the majority of increased cell proliferation would occur during the initial period of estrogen stimulus. At 3 weeks, it would be speculated that the glands had reached a new level of homeostasis.

Bone growth and turnover in 4-OHE_1-treated animals was intermediate between OVX and E_2 treated-animals. Longitudinal growth rate was slowed and both cortical and cancellous turnover data indicate a decrease in the rate of bone formation. Thus 4-OHE_1 attenuated some of the OVX-induced changes but was not as efficacious as E_2. The effects of 4-OHE_1 are contrasted to 2-OHE_1 in the previous study (Westerlind *et al.* 1998) which had no effect on the bone and to 16α-OHE_1 which was nearly as effective as E_2 in antagonizing OVX-induced changes in bone growth and turnover.

It is interesting to note that 4-OHE_1, a catechol estrogen metabolite, has significant estrogen activity whereas its counterpart, 2-OHE_1, has none. This may be a function of the relative binding affinity of the two metabolites for the estrogen receptor. 2-OHE_1 has markedly reduced affinity (2% of estradiol) for the receptor compared with 4-OHE_1 (11% of estradiol) (Martucci & Fishman 1979). The dissociation kinetics of 2-OHE_1 from the estrogen receptor are also much more rapid than 4-OHE_1 (Barnea *et al.* 1983); shorter occupation time would result in a diminished estrogenic response. It is also noteworthy that methylation of C-4 estrogens does not appear to terminate their biological activity as occurs with the 2-hydroxyestrogens (Martucci 1983). Additionally, it has been reported recently that some of effects of the 4-hydroxyestrogens may be non-receptor mediated. Injection of the estrogen antagonist ICI 182780 resulted in abrogation of 17β-estradiol's increase of lactoferrin and progesterone receptor expression in the mouse uterus but had no effect on the increased expression resulting from 4-OHE_2 treatment (Das *et al.* 1998). Activity of the metabolites may also be a function of the absolute or relative presence of estrogen receptors (α, β or other) present in a given tissue.

Although 2-OHE_1 is the major circulating catechol metabolite and the level of 4-OHE_1 is approximately 15–25% of 2-OHE_1 (Adlercreutz *et al.* 1989), it is important to consider that individual tissues have the ability to metabolize estrogens and that circulating levels may not be reflective of the effects at an organ or tissue level (Zhu & Conney 1998). Liehr & Ricci (1996) observed greater 4-hydroxylation in neoplastic breast tissue from breast cancer patients than in breast tissue from woman undergoing reduction mammoplasty. Interestingly, 4-hydroxylase levels were also elevated in the normal breast tissue from the breast cancer patients (Liehr & Ricci 1996). These findings are similar to those that have been observed for 16α-OHE_1. 16α-Hydroxylation was higher in the terminal ductal lobular units in cancerous and non-cancerous tissues from women with breast cancer compared with women without breast cancer (Telang *et al.* 1991, Osborne *et al.* 1993).

Although 4-OHE_1 and 16α-OHE_1 may be more similar in action in the breast, 16α-OHE_1 was profoundly more estrogenic on the bone than 4-OHE_1 – a factor that would suggest its potential role in osteoporosis pathogenesis. Data are beginning to emerge in small clinical studies that elevated 16α-hydroxylation is associated with greater bone mineral density and a reduced rate of bone loss at menopause (Hodge *et al.* 1995, Lim *et al.* 1997).

Table 5 provides a summary of the relative activity of 2- and 16α-OHE_1 (previously evaluated (Westerlind *et al.* 1998)) and 4-OHE_1. The relative estrogen action of each metabolite is expressed as a percentage of OVX'd animals

response to E_2 treatment compared with the vehicle-treated OVX group. The difference in response between the OVX–vehicle and OVX– E_2-treated group is set at 100%. 2-OHE_1 resulted in little to no estrogen action on the measurements evaluated. 4-OHE_1 displayed significant estrogen action on body weight and more moderate estrogen activity in the uterus, mammary gland and bone. 16α-OHE_1 had more marked estrogen action on the bone than either of the other two metabolites and had strong action on body weight. 16α-OHE_1 had moderate estrogenic effects on the uterus and mammary gland.

In conclusion, 4-OHE_1 had tissue-selective activity on the uterus, mammary gland, liver and bone. Its profile of activity was significantly different from the primary circulating catechol metabolite, 2-OHE_1. The differential effects of the two catechol metabolites on estrogen-sensitive tissues warrants consideration as investigators attempt to increase catechol estrogen metabolism to decrease the risk of diseases such as breast cancer.

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Received 18 April 2000
Revised manuscript received 11 July 2000
Accepted 26 July 2000