Differential effects of 17β-estradiol and raloxifene on VSMC phenotype and expression of osteoblast-associated proteins

Ewa Rzewuska-Lech, Muthuvel Jayachandran, Lorraine A. Fitzpatrick, and Virginia M. Miller. Differential effects of 17β-estradiol and raloxifene on VSMC phenotype and expression of osteoblast-associated proteins. Am J Physiol Endocrinol Metab 289: E105–E112, 2005. First published February 15, 2005; doi:10.1152/ajpendo.00366.2004.—Several studies demonstrate an association between osteoporosis and arterial calcific disease, both of which being common in elderly women. Estradiol and raloxifene, a selective estrogen receptor modulator, prevent bone loss in postmenopausal women. Little is known regarding how these agents affect arterial calcification. The aim of this study was to determine whether or not 17β-estradiol and raloxifene reduced vascular smooth muscle cell (VSMC) differentiation and expression of bone-associated proteins during phosphate-induced calcification in vitro. Aortic VSMC were cultured from adult, gonadally intact, and ovariectomized (OVX) female pigs. Calcifying medium was added, and cells were treated with solvent (control), 17β-estradiol (E2), or raloxifene. Extent of calcification and phenotypic expression of bone-associated proteins [matrix gla protein (MGP), osteoprotegerin (OPG), and bone sialoprotein (BSP)] were examined at 3-day intervals over 2 wk. Calcium content increased in all groups but was greater in VSMC derived from intact compared with OVX animals. E2 reduced calcification and preserved a contractile phenotype. Expression of OPG significantly decreased with time; this decrease was significantly greater in VSMC derived from OVX compared with gonadally intact pigs. E2 and raloxifene preserved expression of OPG only in VSMC from intact pigs. Expression of MGP increased significantly with time and was not affected by E2 or raloxifene treatments. E2 treatment significantly inhibited synthesis of BSP in cells from both groups. In conclusion, E2 slows differentiation of VSMC induced by excess phosphate. Effectiveness of raloxifene to preserve expression of bone cell-associated proteins depends on the hormonal status of the tissue donor.

vascular smooth muscle cells; estrogen; raloxifene; selective receptor modulator; smooth muscle phenotype

Arterial calcification is associated with expression of bone- and cartilage-related proteins and endochondryocytes (14, 16, 17, 44). The idea that osteoblast-specific genes could link osteoporosis and arterial calcification is supported indirectly by the high clinical prevalence of arterial calcification and cardiovascular disease in postmenopausal women with osteoporosis (19, 27, 48). An estrogen-replete condition correlated with decreased incidence of coronary arterial calcification in women (12, 52). However, it is not known whether reduced arterial calcification results from effects of estrogen on bone-like characteristics within populations of VSMCs (3, 18).

Insights into common paracrine and autocrine mechanisms contributing to osteoporosis and arterial calcification come from observations of phenotype of genetically altered experimental animals. For example, with loss of the gene for osteoprotegerin (OPG), a modulator of osteoblast differentiation, mice display severe osteoporosis with bone fractures, a phenotype resembling postmenopausal osteoporosis. Paradoxically, these animals present with severe medial arterial calcification by nonendochondral processes (10, 31).

In contrast, mice lacking the gene for matrix gla protein (MGP) display medial calcification of the aorta, which leads to death due to rupture of calcified atheromas, by an entirely endochondral process that may be related to bone matrix protein signaling during postnatal growth (31, 35). MGP is synthesized in bone, cartilage, soft tissues (lung, heart, kidney), and VSMCs (57). Matrix gla protein mRNA synthesis is upregulated in calcified atherosclerotic plaque (50), but it is not known whether estrogen regulates its expression in VSMCs.

Primary cultures of VSMCs express osteoblast-specific genes and proteins (47), including MGP and bone sialoprotein (BSP). BSP binds collagen and is associated with the early phases of bone formation, acting as a potent and specific nucleator of hydroxylapatite (20, 28). BSP is expressed in vein grafts and aortic valves prior to demonstration of overt calcification (13, 44).

Estrogen regulates the synthesis of OPG (26). However, little is known regarding the regulation of osteoblast-associated proteins in VSMCs. Raloxifene, a selective estrogen receptor modulator, is used clinically to treat osteoporosis in women and prevent bone resorption and may reduce adverse cardiovascular events (5). However, direct effects of raloxifene on proteins associated with vascular smooth muscle differentiation and calcification have not been studied. Therefore, experiments were designed to test the hypothesis that 17β-estradiol and raloxifene would decrease vascular smooth muscle trans-

Address for reprint requests and other correspondence: V. M. Miller, Dept. of Surgery, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905 (e-mail: miller.virginia@mayo.edu).

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demonstration, expression of bone matrix proteins, and, therefore, calcification in vitro.

MATERIALS AND METHODS

Chemicals. 17β-Estradiol, β-glycerol, Tris, glycine, sodium orthovanadate, and β-phosphoglycerophosphate were purchased from Sigma Chemical (St. Louis, MO). Raloxifene was purchased from Eli Lilly Pharmaceuticals (Indianapolis, IN). Ready-made polyclonal gels for protein electrophoresis were purchased from Bio-Rad (Hercules, CA). Rabbit anti-rat MGP antibody was a generous gift from Drs. Steve Rannels and Kirk Gilbert from the Pennsylvania State University, Hershey, PA. Monoclonal mouse anti-BSP antibody was purchased from Calbiochem (La Jolla, CA). Monoclonal mouse anti-OPG antibody was purchased from Chemicon International (Temecula, CA). Monoclonal anti-alpha smooth muscle actin antibody, monoclonal anti-desmin antibody, monoclonal anti-vimentin antibody, and anti-human estrogen receptor-β mouse monoclonal IgM were all purchased from Sigma. Anti-calf uterus estrogen receptor-α mouse monoclonal IgG2ak antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell culture. Aortas were obtained from adult (7-mo-old) female pigs (crossbreed of Yorkshire × Landrace × Hampshire × Duroc) with intact ovaries and adult female pigs that had been ovarioctomized for 8 wk (OVX). Pigs reach sexual maturity between 4 and 6 mo of age (11), and estrogen is undetectable in serum of OVX pigs (4). VSMCs were cultured from the aorta by means of an explant technique as previously described by our group (8). Briefly, a fragment of aorta was stripped of intima and adventitia. The remaining medial layer was cut into small pieces and placed in growth medium [phenol red-free medium 199 (M199, Sigma) with 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA)] in multiwell culture plates. After 5–7 days, cells migrated from the explants, and explant fragments were removed after ~15 days of culture. The VSMC phenotype was confirmed by positive immunostaining for α-actin, desmin, vimentin, and myosin heavy chain. Cells were cultured at 37°C in a humidified atmosphere of 95% O2-5% CO2 using M199 medium, which was phenol red free, supplemented with 10% heat-inactivated fetal bovine serum (Biofluids-Biosource, Camarillo, CA), antibiotics (penicillin-streptomycin; Gibco, Carlsbad, CA) and antimycotic (Fungizone, Gibco). Cells at passages 2 and 3 were used for experiments.

VSMCs do not spontaneously calcify in culture (3). Therefore, at ~80% confluence, cells were placed into calcifying medium consisting of the growth medium described above supplemented with 10 mM β-glycerophosphate (6). That day was called day 0 for all experiments. Treatment consisted of 17β-estradiol at a final concentration 10−9 M/l, raloxifene at 10−9 M/l final concentration, and control group, which received solvents (10−9 M/l DMSO) used for estradiol and raloxifene. These concentrations of raloxifene and estradiol were chosen on the basis of published literature for efficacy at physiological levels (56). Medium was changed every 3 days. Cells were collected on days 0, 1, 2, 3, 6, 9, 12, and 15 for determination of calcium content and protein expression.

von Kossa staining. To confirm the presence of calcification nodi and the presence of calcium phosphate deposits, cells were grown in slide chambers (Fischer) and then stained with silver nitrate. The presence of black stain confirmed the presence of calcium phosphate salts. The slides were counterstained with hematoxylin.

Calcium content. At each designated time point, cells were incubated in 0.6 N HCl for 1 h. The supernatant was tested for calcium content colorimetrically by the o-cresolphthalein complexone method (Sigma). The remaining cells were scraped and lysed. Total protein content was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Total calcium content was normalized to total protein content for each culture.

Western blotting. At each designated time point, cells were twice washed with PBS and lysed in buffer (1% SDS, 1 mM sodium vanadate, 10 mM Tris, pH 7.4). The lysate was passed through a 26-gauge needle, briefly sonicated, and centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant was collected. Total protein content was determined by the BCA method. Fifty micrograms of total protein from each sample were loaded onto polyacrylamide gel (Bio-Rad) and separated by electrophoresis. The protein transfer was performed by the semidyrid method (Bio-Rad) onto Hybond-supported nitrocellulose membrane (Amersham International, Buckinghamshire, UK) and polyvinylidene difluoride (PVDF) membrane. The blot was then blocked in 5% blocking buffer (Bio-Rad) in TBS-T (Tris-buffered saline + 0.1% Tween 20, Bio-Rad) for a minimum of 1 h. The membrane was incubated with the primary antibodies (actin 1:500, desmin 1:400, vimentin 1:400, MGP 1:5,000, BSP 1:1,500, and OPG 1:1,000) at room temperature for 1 h. The membrane was washed three times in TBS-T and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). Detection was performed with a chemiluminescence detection kit according to the manufacturer’s protocol (ECL Detection, Amersham International). The time of film exposure was standard for each protein. For expression of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), proteins were transferred onto PVDF membrane, and nonspecific protein expression was blocked with 5% nonfat dry milk (Bio-Rad) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol) for 1 h. Membranes were incubated at 4°C with primary anti-ERα IgG antibody (1:500 dilution in transfer buffer) or anti-ERβ IgM antibody (1:500 dilution in transfer buffer) overnight. These membranes, incubated with primary antibodies, were washed twice (5 min in each) in 1× TBS (Bio-Rad) and treated with specific secondary goat anti-mouse-HRP conjugates (50 μl in 10 ml of 1× TBS) for 2 h at room temperature. Protein expression on membranes was determined by a colorimetric method using an Opti-4CN Substrate kit (Bio-Rad). Opti-4CN substrate was freshly prepared according to the manufacturer’s instructions. Specific protein bands on films (chemiluminescence detection) and membranes (colorimetric determination) were scanned and digitized by positive segment analysis using UN-SCAN-IT (30). An equal amount of proteins transferred from gel to membrane was determined by β-actin expression (29). Average pixel values from each individual blot were used for statistical analysis.

Statistical analysis. The least squares method was used for statistical analysis of changes in markers of smooth muscle differentiation between day 0 and day 15. Difference between slopes of trend line for each group was analyzed using a paired Student’s t-test. As total protein decreased with extent of calcification, values of band intensity for OPG, MGP, and BSP at day 12 were expressed as percentages of initial values at day 0. The Mann-Whitney rank sum test was used to compare these data. Differences among treatment groups were analyzed using one-way analysis of variance. Further analysis was based on Tukey’s test or Bonferroni pairwise comparisons. P values of <0.05 were considered significant. Experiments were conducted in duplicate using cells from three different animals/groups.

RESULTS

VSMCs at day 0 (prior to exposure to medium containing excess phosphate) were confluent and spindle shaped (Fig. 1A). In response to addition of the calcifying medium, cells formed dendrite-like processes and extracellular nodules that stained for calcium phosphate with von Kossa stain (Fig. 1, B and C). Calcium content of the cultures began to increase at day 6 and rose throughout the remainder of the experiments (Fig. 2). At day 15, under control conditions, calcium accumulation was statistically greater in cells derived from intact compared with OVX animals. Treatment with 17β-estradiol significantly re-
duced calcium accumulation in cells derived from intact animals. Development of calcification was similar in cells treated with raloxifene and 17β-estradiol in this group. However, in cultures derived from OVX animals, calcium accumulation was significantly less in cells treated with 17β-estradiol than in cells treated with raloxifene (Fig. 2). There was a significant decrease in total protein of all cultures at day 15, when calcification was the greatest.

To determine whether changes in VSMC phenotype preceded calcification, protein analyses were performed on cultures at day 0, 1, 2, 3, 6, 9, and 12. During this time, neither calcium content nor total protein differed among groups. There were no differences in expression of α-actin, desmin, or vimentin (proteins characteristic of a contractile phenotype) among cultures obtained from intact or OVX pigs prior to exposure to the calcification media (day 0; Fig. 3). There was a progressive linear decrease in expression of α-actin, desmin, and vimentin when VSMCs were exposed to the calcification medium over the 12 days preceding significant calcification. Decreases in expression of these markers of smooth muscle phenotype were similar in cultures derived from aorta of intact and OVX pigs (Fig. 3 and Table 1).

Expression of OPG was determined at each of the five designated time points throughout the 2-wk experiment. OPG expression showed a significant and time-dependent linear decrease in expression in cells from both groups of pigs (slope = −3.79 with \( r^2 = 0.96 \) and slope = −5.08 with \( r^2 = 0.98 \) for intact and OVX animals, respectively), reaching a statistically significant difference in expression at day 12 (Fig. 4). Both 17β-estradiol and raloxifene preserved expression of OPG in cells derived from intact pigs (Fig. 4). Neither treatment had a significant effect on expression of OPG in cells derived from OVX animals (Fig. 4).

Expression of MGP increased in cells from both intact and OVX pigs exposed to the calcifying medium. The increase occurred within the first 3 days of exposure to the calcifying medium, was not different between cells derived from intact and OVX pigs, and was not affected by exposure to either 17β-estradiol or raloxifene (data not shown).

Expression of BSP did not change significantly in control cultures from either intact or OVX pigs during the experiment (Fig. 5). However, in cells from both intact and OVX pigs,
of neither ERα nor ERβ was statistically different from expression at day 0 (Fig. 6). However, when expression of ERα was compared with expression of ERβ, statistically significant differences were observed. Specifically, in cells from intact animals, expression of ERα was significantly less than that of ERβ with treatment of both 17β-estradiol and raloxifene \( (P < 0.05) \); whereas in cells derived from OVX animals expression of ERα was significantly less than that of ERβ only in the presence of raloxifene \( (P < 0.01; \text{Fig. 6}) \).

### DISCUSSION

Results of this study demonstrate that 17β-estradiol preserves expression of proteins characteristic of a contractile phenotype (α-actin, desmin, and vimentin) in VSMCs exposed to a calcifying medium to a greater extent than the SERM raloxifene. Furthermore, 17β-estradiol preserved expression of OPG and decreased expression of BSP, both proteins associated with osteoblasts. The combined effects in the expression of these two proteins would be to reduce calcification. Another important observation is that the ability of both 17β-estradiol and raloxifene to alter the phenotype of VSMCs is dependent on the hormonal status of the animals from which cells were derived. These results have important implications toward understanding how hormone therapy could reduce arterial calcification postmenopause (52).

Although the phenotype of VSMCs changes during cell culture, calcification does not proceed spontaneously (37). However, subpopulations of VSMCs, when exposed to transforming growth factor-β and cholesterol, will form calcific nodules, a process facilitated by estrogen treatment, after ~2 wk in culture (3). Cultures used in the present study were not preselected for their potential to form calcified nodules. In these experiments, β-glycerophosphate was used to “accelerate” the calcification process. 17β-estradiol reduced the transdifferentiation of the cells to a calcifying phenotype. Although the total number of cells and the initial expression of α-actin, desmin, and vimentin were not different between cells derived from intact and ovariectomized animals, other characteristics not measured in this study may phenotypically differentiate the cultures. Indeed, cultures were developed by explant technique rather than by enzymatic dissociation, and it is possible that the migratory potential of cells within the donor tissue differed (18). Because 17β-estradiol and raloxifene did not modulate protein expression to the same extent in both populations of cells indirectly supports this conclusion. However, if hormone depletion modulated cell populations within the medium, then

17β-estradiol, but not raloxifene, significantly reduced expression of BSP (intact: slope \( = -1.53, r^2 = 0.77; \text{OVX: slope} = -2.08, r^2 = 0.65; \text{Fig. 5})\).

Expression of estrogen receptors (ERα and ERβ) was not different in cultured aortic VSMCs from gonadally intact and OVX pigs prior to exposure to the calcifying medium (day 0). After exposure to the calcifying medium (day 12), expression

![Graph showing changes in expression of α-actin, desmin, and vimentin during the time course of experiment. Expression of all 3 proteins decreased in response to addition of calcifying medium. Changes in expression were defined by linear least square regression. Slopes and \( r^2 \) are reported in Table 1. Data are shown as means ± SE of cells derived from 3 different animals per treatment group. Dotted line represents statistical prediction.](http://ajpendo.physiology.org/)

**Table 1.** Slopes for rates of decline in protein expression in pig aortic smooth muscle after 12-day exposure to calcifying medium

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Control</th>
<th>Estradiol</th>
<th>Raloxifene</th>
</tr>
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<tr>
<td>Actin slope</td>
<td>-2.60</td>
<td>-1.08†</td>
<td>-1.58†</td>
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<td>( r^2 )</td>
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<td>0.95</td>
<td>0.85</td>
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<td>Desmin slope</td>
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<td>-1.97†</td>
<td>-2.66</td>
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<tr>
<td>( r^2 )</td>
<td>0.92</td>
<td>0.98</td>
<td>0.92</td>
</tr>
<tr>
<td>Vimentin slope</td>
<td>-1.87</td>
<td>-1.01†</td>
<td>-2.23</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.97</td>
<td>0.74</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Values are calculated as slope of line defining change in protein expression from day 0 to 12 days after exposure of cells to calcifying medium (see Fig. 3). Least square method was used to define the lines. †Statistical difference of treatment vs. control cultures within each group (i.e., cells derived from intact or ovariectomized pigs). *Statistically significant difference in slope with raloxifene treatment between cells derived from ovariectomized vs. intact, \( P < 0.05 \).

![Graph showing changes in expression of α-actin, desmin, and vimentin during the time course of experiment. Expression of all 3 proteins decreased in response to addition of calcifying medium. Changes in expression were defined by linear least square regression. Slopes and \( r^2 \) are reported in Table 1. Data are shown as means ± SE of cells derived from 3 different animals per treatment group. Dotted line represents statistical prediction.](http://ajpendo.physiology.org/)
similar results would occur regardless of the technique used to derive the primary smooth muscle culture. Evidence suggests that the vascular wall changes phenotypically after hormone depletion. In clinical trials, hormone treatment administered in the immediate postmenopausal period reduced coronary calcification (12, 52), whereas initiation of hormone treatment late in the menopause was without benefit to prevent adverse cardiovascular events (21, 23, 24).

Exposure of VSMCs to a calcifying medium, in addition to differences in treatment effects on expression of smooth muscle contractile phenotype, affected expression of bone cell-associated proteins. In particular, in the absence of hormone treatment, expression of OPG decreased, MGP increased, and BSP was unchanged in VSMCs derived from either ovary-intact or ovariectomized animals.

OPG inhibits vitamin D- and warfarin-induced calcification of cells in culture (42). Therefore, it is possible that decreases in OPG would facilitate the process of calcium incorporation, an observation supported by the results of the present study. In addition, whether or not 17β-estradiol promotes OPG synthesis in bone is dependent on hormonal status and coexisting diseases (33). Consistent with these concepts, expression of OPG was significantly dependent on hormonal status of donor animals such that raloxifene and 17β-estradiol preserved synthesis (reduced the loss) of OPG in cells from animals with ovaries but not in ovariectomized animals. These changes are consistent with reduced calcification in cells from intact animals and are consistent with expression of OPG by immunostaining with calcification of human aortic valves (31). Mechanisms, (i.e., activation of receptor activator of NF-κB ligand or osteoblast transcription regulators such as Msx2 and Cbfα1/Runx2) by which 17β-estradiol or raloxifene regulates OPG in VSMCs, remain to be determined (31, 54).

In the present experiments, no significant differences were observed in the expression of MGP among 17β-estradiol-, raloxifene-, or solvent-treated aortic smooth muscle cells. In all three groups, expression of MGP significantly increased throughout the duration of the experiment. This observation is in accord with earlier reports (43). Regulation of MGP by estrogen may be cell specific, as 17β-estradiol upregulated MGP transcription within 48 h of exposure in human breast...
cancer cells (51). In addition, differences in regulation of MGP and OPG by estrogen and raloxifene are consistent with differences in endochondral processes characteristic of medial calcification observed in OPG and MGP knockout mice (31).

BSP binds collagen and can nucleate hydroxylapatite, even in a cell-free in vitro model system (agarose gels) (28). Although BSP can be induced in VSMCs by factors that promote their differentiation toward an osteoblastic phenotype, unstimulated cells express minimal or no BSP (36). In clinical studies, serum BSP was significantly elevated in postmenopausal women with osteoporosis (49) and is inversely related to serum estradiol levels in women (38). Serum BSP decreased after antiresorptive treatments (hormone replacement therapy or bisphosphonate), parallel to a decrease of bone resorption markers. Thus it has been proposed that serum BSP be used as a marker of noncollagenous organic bone matrix in assessment of bone turnover (49). The decline in BSP expression during 17β-estradiol treatment of cells in the present study is consistent with observations in human vascular tissue, ERα seems to be the predominant ER in porcine aortic smooth muscle (25). How each receptor subtype may be up- or down-regulated with various atherosclerotic disease processes is unclear (1, 34). Results from the present study suggest that both ERs may be regulated during the process of calcification. Changes in the ratio of ERα and ERβ may be important in regulating gene transcription, as the two ERs can form both homo- and heterodimers (2, 58). However, nongenomic effects of 17β-estradiol and raloxifene mediated through dimers of ERs cannot be ruled out at this time and could include direct effects on calcium channel activation and rapid activation of second messenger signaling pathways like cAMP, extracellular signal-related kinase, and phosphatidylinositol 3-kinase (39, 45). Because both 17α-estradiol and the receptor antagonist ICI 182,780 have partial agonist activity in pig tissue (Refs. 7 and 39, and unpublished observations by our group), it was not possible to determine whether effects of the hormone treatments were mediated by mechanisms other than those associated with ER activation. Differences in efficacy between effects of 17β-estradiol and raloxifene could be metabolites of 17β-estradiol (15). Determining the relative contribution of

![Fig. 6. Representative Western blots and changes in densitometry measurements (average pixel values) of estrogen receptor-α (ERα; top) and ERβ (bottom) in cells from gonadally intact (left) and OVX pigs (right) exposed to medium supplemented with 10 mM β-glycerophosphate in the presence of solvent (control, DMSO; 10−6 m/l), E2 (10−6 m/l), or raloxifene (10−6 m/l). Data are shown as means ± SE of %increase from day 0. Duplicate experiments were conducted on cells derived from 3 different animals per treatment group. *Statistically significant difference in expression of ERα vs. ERβ in cells from intact or OVX animals treated with E2 or raloxifene by ANOVA followed by Bonferroni multiple comparison, P < 0.05.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00754.2004)
ERα compared with ERβ awaits the development of more selective and specific ER ligands or inhibitors or examination of vascular smooth muscle cultures derived from animals with selective deletion of ERs.

In conclusion, treatment of cultured VSMCs with 17β-estradiol slows transdifferentiation induced by a calcifying medium and is manifested by maintaining expression of α-actin, desmin, vimentin, and OPG and decreasing expression of BSP. However, the magnitude of the effects of 17β-estradiol and of raloxifene, the selective estrogen receptor modulator in mimicking responses of 17β-estradiol, is dependent on the hormonal status of the tissue donor. These differences may be related to regulation and activation of estrogen receptor-associated/dependent pathways.

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Current address of L. A. Fitzpatrick: Director Global Development, Inflammation TA, Amgen, One Amgen Center Dr., Mail Stop 38-2-B, Thousand Oaks, CA 91320-1799.

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