Preferential Growth Stimulation of Mammary Glands over Uterine Endometrium in Female Rats by a Naturally Occurring Estradiol-17β-fatty Acid Ester

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ABSTRACT

We hypothesize that the endogenously present lipoidal estrogen fatty acid esters may have a stronger mitogenic action in the fat-rich mammary tissues than in the uterus. To test this hypothesis, we compared the activity of estradiol-17β-fatty acid esters (E2-17β-S) with that of estradiol-17β (E2) in stimulating the growth of mammary glandular cells versus the growth of uterine endometrial cells in ovariectomized female Sprague Dawley rats. Experimentally, an estimated 0.5 or 5 nmol of E2-17β-S or E2 was released daily to ovariectomized female rats through an Alzet pump implanted under the back skin of the animal for 10 or 23 days. The growth-stimulatory effect of E2-17β-S and E2 on mammary glandular cells was determined according to 5-bromo-2'-deoxyuridine labeling indices, and their effect on the uterus was determined by measuring both the 5-bromo-2'-deoxyuridine labeling index and the uterine wet weight. Our results showed that chronic treatment of ovariectomized female rats with 0.5 or 5 nmol/day E2-17β-S for 10 or 23 days had a stronger stimulatory effect on mammary glandular cell proliferation than treatment with equimolar doses of E2. In the uterus, however, E2 was more active in stimulating the proliferation of uterine endometrial cells than E2-17β-S at equimolar doses. Our results demonstrated, for the first time, that a naturally occurring estradiol-17β-fatty acid ester has a differential, strong mitogenic effect in the fat-rich mammary tissues, and this effect was not observed with E2. It is tempting to suggest that the fatty acid esters of the endogenous estrogens and their bioactive metabolites (e.g., 4-hydroxyestriadiol and 16α-hydroxyestriol) may be of unique importance for stimulating cell growth and possibly also for inducing tumor formation in the fat-rich mammary tissues as compared with the uterus. More studies are warranted to test these ideas.

INTRODUCTION

It has been known for decades that chronic administration of an estrogen induces tumor formation in the target organs of a number of laboratory animals (1–2). In humans, there is strong evidence associating chronic estrogen administration with an increased risk of postmenopausal uterine endometrial cancer (8–12). Although there is also mounting evidence associating elevated human breast cancer risk with an increased total lifetime exposure to estrogens (13–16), this relationship generally is not as strong as the link between estrogen and human endometrial cancer. The mechanism underlying the difference is not fully understood at present. Zhu and Conney (17) recently pointed out that measurement of the circulating unmetabolized parent estrogens, such as E2, estrone, and estradiol-17β-fatty acid ester, with E2 for 10 or 23 days had a stronger stimulatory effect on mammary glandular cell proliferation than treatment with equimolar doses of E2. In the uterus, however, E2 was more active in stimulating the proliferation of uterine endometrial cells than E2-17β-S at equimolar doses. Our results demonstrated, for the first time, that a naturally occurring estradiol-17β-fatty acid ester has a differential, strong mitogenic effect in the fat-rich mammary tissues, and this effect was not observed with E2. It is tempting to suggest that the fatty acid esters of the endogenous estrogens and their bioactive metabolites (e.g., 4-hydroxyestriadiol and 16α-hydroxyestriol) may be of unique importance for stimulating cell growth and possibly also for inducing tumor formation in the fat-rich mammary tissues as compared with the uterus. More studies are warranted to test these ideas.

Received 1/26/01; accepted 5/31/01.

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2 The abbreviations used are: E2, estradiol-17β; E2-17β-S, estradiol-17β-fatty acid ester; PRL, prolactin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; RIA, radioimmunoassay; BrdUrd, 5-bromo-2'-deoxyuridine.
MATERIALS AND METHODS

Chemicals. \( E_2 \)-17\( \beta \)-S and \( E_2 \) were obtained from Steraloids (Newport, RI). Our high-performance liquid chromatography analysis of \( E_2 \)-17\( \beta \)-S showed a purity of almost 100%. BrdUrd, 3,3′-diaminodibenzidine (as the peroxidase substrate, in a tablet set), acidic alcohol, and Scott’s tap water were purchased from the Sigma Chemical Co. (St. Louis, MO). Mouse anti-BrdUrd primary antibody was obtained from Novocastra Laboratory (New Castle upon Tyne, United Kingdom). The Vector ABC Elite kit, 1% normal horse serum, and biotinylated secondary antibody against mouse IgG were obtained from Vector Laboratories (Burlingame, CA).

Animal Experiments. Ovariectomized female Sprague Dawley rats (average age, 6.5 weeks old) were purchased from Harlan Sprague Dawley Laboratory (Houston, TX). After arrival, they were allowed to acclimatize for a week before the experiment began. The animals were housed under controlled conditions of temperature and photoperiod (12-h light/12-h dark cycle) and had free access to food and water throughout the experiment period. For the first set of experiments, the animals were randomly divided into one control group and two treatment groups (one \( E_2 \)-17\( \beta \)-S treatment group and one \( E_2 \) treatment group). Each animal in the \( E_2 \)-17\( \beta \)-S or \( E_2 \) treatment group was surgically implanted under the back skin with an Alzet osmotic minipump (model 1002; Alza Scientific Products, Palo Alto, CA) with a syringe by following the supplier’s instructions. The Alzet minipumps were then surgically implanted under the back skin of each ovariectomized female rat under halothane anesthesia, a procedure approved by the Institutional Animal Care and Use Committee of the University of South Carolina.

For the second experiment, the same procedure was followed, except five groups of animals were used: one control group, two \( E_2 \)-17\( \beta \)-S treatment groups, and two \( E_2 \) treatment groups. The Alzet minipumps were filled with a 0.083 or 0.83 mM solution of an estrogen, which was estimated to release 0.5 and 5 nmol/day \( E_2 \)-17\( \beta \)-S or \( E_2 \). In this animal experiment, a larger size of the Alzet minipump (model 2004) was used, and these pumps would continuously release the filled solution at a rate of 0.25 \( \mu \)l/day for up to 28 days. The procedure for preparation of the minipumps was the same as that described above for the first experiment. All of the animals used in this experiment were sacrificed at 23 days after implantation of the Alzet minipumps.

BrdUrd Labeling of Proliferating Cells in the Breast and Uterus of Female Rats. The BrdUrd labeling of proliferating cells in the breast and uterus of female Sprague Dawley rats was carried out as described previously (36). Two h before sacrifice, the animals were injected i.p. with 50 \( \mu \)g BrdUrd/g body weight (dissolved in sterile saline). The animals were then euthanized in a small CO\(_2\) chamber and immediately decapitated. Blood samples (average, 4–5 ml) were collected from each animal and placed in Vacutainer test tubes containing heparin sodium (purchased from Fisher Scientific, Suwanee, GA). The uteri were removed, trimmed of excess connective tissues, and weighed precisely to determine their wet weight. The mammary tissues were also removed. The mammary and uterine tissues were fixed in Carnoy’s fixative for 3 h and dehydrated by sequential transfer through 7-\( \mu \)m sections, and placed on Superfrost microscope slides (Fisher Scientific). The sections were then placed in xylene to remove paraffin and rehydrated by transfer through a gradient of ethanol solutions with decreasing concentrations. Immediately after this step, the slides were placed in a solution of 3% H\(_2\)O\(_2\) (in methanol) to quench endogenous peroxidase activity and then treated with 4.0 N HCl for 30 min to denature the cellular DNA. The sections were blocked using 1% normal horse serum and then incubated with anti-BrdUrd primary antibody (Novocastra Laboratory) at 1:100 dilution for 60 min, followed by incubation with a 1:100 dilution of a biotinylated secondary antibody (Vector Laboratories) for 45 min at room temperature. Detections of BrdUrd-incorporated cells were done by using ABC Elite reagent (Vector Laboratories), and color was developed by incubation with 3,3′-diaminodibenzidine for 5 min. Harris hematoxylin was used as a counterstain, and the slides were preserved with Cytoseal (Stephens Scientific, Kalamazoo, MI). Typically, the large nuclei of the BrdUrd-incorporated cells were selectively stained brown (sometimes dark brown). The numbers of the stained and unstained mammary glandular cells or uterine endometrial cells for each tissue slide were determ-
mined manually by counting ~10 representative fields under a light microscope (×40) in a double-blinded manner, and the BrdUrd labeling indices (the percentage of cells positively stained) were then calculated accordingly.

RIA of the Plasma Levels of E2, PRL, LH, and FSH. The collected whole blood sample from each animal was kept in a Vacutainer test tube at 4°C for 2 h and then centrifuged for 15 min at 3000 rpm. Aliquots (200 μl) of the plasma samples were transferred precisely to another set of small vials with sealed caps and stored at −80°C. The plasma levels of E2 were measured by using a double-antibody RIA kit provided by Diagnostic Products Corp. (Los Angeles, CA). Immediately before the assay, each plasma sample was diluted in the zero calibrator buffer provided by the vendor. Known standards, also provided by the vendor, and the rat plasma samples were incubated with 100 μl of rabbit anti-E2 antibody for 2 h at room temperature followed by a 1-h incubation with 100 μl 125I-labeled E2. Aliquots of the ice-cold precipitating solution were then added to each tube, and all tubes were centrifuged for 15 min at 3000 × g. The supernatant was decanted, and the sediment in each tube was measured for radioactivity with a gamma counter. The concentration of E2 in the unknown samples was calculated according to the standard curve generated. The assay sensitivity for E2 was 5 pg/ml.

The plasma levels of rat LH, FSH, and PRL were determined by highly sensitive, specific, and quantitative RIAs, using the RIA reagent sets prepared and distributed by the National Hormone and Peptide Program of the NIDDK, directed by Dr. A. F. Parlow (Parlow@humc.edu). Results were expressed as ng/ml, in terms of rat LH (RP-3), FSH (RP-2), and PRL (RP-3).

RESULTS

Effect of E2-17β-S versus E2 on Uterine and Mammary Cell Growth

Experiment I. In this initial experiment, an estimated 5 nmol/day E2-17β-S or E2 was released to ovariectomized female Sprague Dawley rats for 10 days through an Alzet minipump implanted under the back skin of each animal at ~7.5 weeks of age. The growth-stimulatory effect of E2-17β-S and E2 on mammary glandular cells was determined by measuring the BrdUrd labeling index, and their effect on the uterus was determined by measuring both the uterine wet weight and the BrdUrd labeling index for uterine endometrium.

As shown in Fig. 2A, treatment of animals with 5 nmol/day E2-17β-S or E2 for 10 days significantly increased the uterine wet weight as compared with that of the controls, but the activities of these two estrogens were not significantly different. However, the rate of uterine endometrial cell proliferation (according to the BrdUrd labeling in-
Dex) in animals treated with 5 nmol/day E2-17β-S or E2 was found to be differentially increased by 2.6-fold (P, 0.02) or 16.3-fold (P, 0.001), respectively, over the controls (Figs. 2B and 3, A–C). These data suggested that treatment of ovariectomized female rats with 5 nmol/day E2 had a much stronger stimulatory effect on the proliferation of uterine endometrial cells than did 5 nmol/day E2-17β-S.

In comparison, the rate of mammary glandular cell growth (according to the BrdUrd labeling index) after 10 days of treatment with 5 nmol/day E2-17β-S or E2 was increased by 5.2-fold (P < 0.001) or 1.6-fold (P < 0.01), respectively, over the controls (Figs. 2C and 3, D–F). These data suggested that E2-17β-S had a stronger growth-stimulatory effect on mammary glandular cells than did E2, which was exactly opposite to what was observed in the uterus.

**Experiment II.** To confirm and also to extend these findings, we further compared the mitogenic action of E2-17β-S versus E2 in the breast and uterus at two dose levels (0.5 and 5 nmol/day) and for a longer treatment period (23 days). These two daily dose levels were used because several earlier studies showed that 5 nmol/day E2 produced a near maximum uterotrophic response and that 0.5 nmol/day E2 would generate a ~50% maximum response in ovariectomized young female rats or in immature female rats. In this experiment, E2-17β-S and E2 were administered using larger-sized Alzet minipumps (model 2004) that were also implanted under the back skin of each animal at ~6 weeks of age.

Similar to the results from the first experiment, the uterine wet weight of each animal treated with either 0.5 or 5 nmol/day E2-17β-S or E2 for 23 days was significantly increased as compared with that of the controls (see Fig. 4, A and A'). At the lower dose level (0.5 nmol/day), the stimulatory effect of E2 on uterine wet weight gain was greater than that of E2-17β-S (P < 0.001, Fig. 4A), but their effects were not different at the higher dose (5 nmol/day; Fig. 4A'). Measurement of the BrdUrd labeling indices showed that 0.5 nmol/day E2 stimulated the proliferation of uterine endometrial cells ~5 times more than did E2-17β-S (P < 0.001; Fig. 4B). A 10× higher dose (5 nmol/day) of E2 had ~30% higher activity in stimulating the proliferation of endometrial cells than the lower dose; in contrast, the higher dose of E2-17β-S had ~300% higher activity than the lower dose (compare Fig. 4, B and B'). These data suggested that E2 had a higher potency than E2-17β-S in stimulating uterine endometrium growth.

According to the BrdUrd labeling index, the rate of mammary glandular cell growth in untreated ovariectomized female rats was 1.1 ± 0.1% (Fig. 4, C and C'). In animals treated with 0.5 nmol of E2-17β-S or E2 for 23 days, the rate of mammary cell growth was increased to 3.1 ± 0.2% (200% increase over the control; P < 0.001) or 2.0 ± 0.2% (90% increase over the control; P < 0.01), respectively.
showed higher plasma concentrations of free E₂ than control animals receiving no estrogen treatment, but the differences between E₂-17β-S- and E₂-treated animals were not statistically significant (Table 1). Our measurements of the plasma levels of free E₂ concentrations in the control and E₂-treated animals were in close agreement with an earlier report (37).

Chronic administration of 0.5 nmol/day E₂ for 23 days showed a stronger negative regulatory effect on the plasma LH levels than did administration of 0.5 nmol/day E₂-17β-S (Table 1). However, both of the estrogens markedly decreased the plasma LH levels to a similar extent at a 10× higher dose (5 nmol/day; Table 1). The plasma levels of FSH in ovariectomized female rats were not significantly affected by treatment with 0.5 or 5 nmol/day E₂-17β-S or E₂ for 23 days (Table 1). These data suggested that the pituitary LH and FSH secretion had different sensitivities to estrogen feedback regulation. Analysis of the plasma levels of PRL indicated that chronic administration of 0.5 nmol/day E₂-17β-S or E₂ did not show detectable effects on the plasma levels of PRL. A 10× higher dose (5 nmol/day) of both estrogens markedly increased the plasma PRL levels, but their effects were not significantly different from each other (Table 1).

In summary, administration of 0.5 or 5 nmol/day E₂ for 23 days showed a somewhat stronger inhibitory effect on the animal’s body weight gain than did E₂-17β-S. Chronic administration of 0.5 nmol/day E₂ was significantly more repressive than an equimolar dose of E₂-17β-S in lowering the postovariectomy plasma levels of LH. However, this difference was not evident either in relation to their plasma PRL-elevating activity or in relation to their virtual noneffect in repressing plasma FSH levels.

**DISCUSSION**

In the present study, we compared the mitogenic effect of E₂-17β-S with E₂ in the breast and uterus of ovariectomized female rats. Our results showed that chronic administration of 0.5 or 5 nmol/day E₂-17β-S to ovariectomized female rats for 10 or 23 days had a markedly stronger stimulatory effect on mammary glandular cell proliferation than did E₂ at equimolar doses. In the uterus, however, E₂ was much more active than E₂-17β-S in stimulating the proliferation of endometrial cells.

**Effect of E₂-17β-S versus E₂ on Animal Body Weight and Plasma Levels of E₂, PRL, FSH, and LH**

It is known that chronic estrogen administration decreases an animal’s body weight gain in a dose-dependent manner (37). Our results also showed that administration of 0.5 or 5 nmol/day E₂-17β-S or E₂ for 23 days decreased the animal’s body weight gain. Furthermore, the body weights of the animals treated with 0.5 or 5 nmol/day E₂ were somewhat lower than those receiving equimolar doses of E₂-17β-S (Fig. 5).

We also determined the plasma levels of free E₂, PRL, FSH, and LH in ovariectomized female rats treated with 0.5 or 5 nmol/day E₂-17β-S or E₂ for 23 days. Analysis of plasma levels of free E₂ by RIA showed that animals receiving 0.5 or 5 nmol/day E₂-17β-S or E₂ showed a more stimulatory effect on mammary glandular cell proliferation than did E₂ at equimolar doses. In the uterus, however, E₂ was much more active than E₂-17β-S in stimulating the proliferation of endometrial cells.

**Table 1 Effect of chronic E₂-17β-S or E₂ administration (0.5 and 5 nmol/day for 23 days) on the plasma levels of free E₂, LH, FSH, and PRL in ovariectomized female rats**

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² p < 0.01 as compared to the corresponding control.

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enous estrogen-fatty acid esters were present in the blood, but much higher concentrations were present in the fat tissues (24). Because the mammary glandular cells are surrounded by large amounts of adipocytes when compared with uterine endometrial cells, it is possible that the large amounts of mammary adipocytes may serve as a storage site for the endogenously formed estrogen fatty acid esters, which may partially contribute to the stronger mitogenic activity of E2-17β-S in mammary glands compared with the uterus.

It is known that the estrogen fatty acid esters themselves cannot activate the estrogen receptor. Their potent, long-lasting hormonal activity in the target organs results largely from the slow in situ release of the parent estrogens through esterase-mediated cleavage of the fatty acid chain (18). The differential mitogenic activity of E2-17β-S in the mammary glands observed in this study may also be due to the presence of higher estrogen esterase activity in the breast tissues as compared with the uterus, which would release more bioactive estrogens from their respective estrogen fatty acid esters for stimulation of mammary cell growth. In support of this speculation, an earlier study showed the presence of estrogen esterase activity in human breast cancer cells (38). Our recent preliminary studies also showed that high estrogen esterase activity was present in rat mammary crude homogenates and microsomes, and this esterase activity was markedly higher than that in the uterus. More research is warranted to advance our knowledge on the tissue/cell distribution of estrogen esterase activity and its regulation by hormonal and environmental factors.

It should be noted that several earlier studies have compared the uterotrophic effect of a few endogenously present estrogen fatty acid esters with that of E2 when given to female animals (18, 19, 21). In most of these studies, the immature female rats or mice usually received a single s.c. or i.v. injection of an estrogen-fatty acid ester or E2 (in oil solutions), and the uterotrophic activity was determined by measuring uterine wet weight at various time points after estrogen injection (18, 19, 21). The results of these studies showed that the estrogen fatty acid esters had a longer-lasting and stronger stimulatory effect than E2 on uterine wet weight increase (18, 19, 21). Although similar doses or the same doses (such as 5 nmol/day) of the estrogen fatty acid esters were also used in some of the earlier studies, the routes of administration were different: whereas we gave the estrogen by using minipump-controlled continuous release, most of the earlier studies gave the total amount of an estrogen with a single i.p. or i.v. injection. Comparing these two common methods of estrogen administration, we believe that the minipump-controlled continuous release may bear a greater resemblance to endogenous hormone secretion. This difference in the route of estrogen administration is likely a major contributing factor to the observed differences in the biological activity in the uterus.

Lastly, it is also of interest to note that our results indicated that the uterine wet weight gain in female rats treated chronically with an estrogen did not fully agree with the rate of uterine endometrial cell proliferation as determined by measuring the BrdUrd labeling indices. Assaying the uterotrophic activity in mice or rats by measuring uterine wet weight increase (18, 19, 21). Although similar doses or the same doses (such as 5 nmol/day) of the estrogen fatty acid esters were also used in some of the earlier studies, the routes of administration were different: whereas we gave the estrogen by using minipump-controlled continuous release, most of the earlier studies gave the total amount of an estrogen with a single i.p. or i.v. injection. Comparing these two common methods of estrogen administration, we believe that the minipump-controlled continuous release may bear a greater resemblance to endogenous hormone secretion. This difference in the route of estrogen administration is likely a major contributing factor to the observed differences in the biological activity in the uterus.

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Cancer Res 2001;61:5764-5770.

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