Estradiol Release Kinetics Determine Tissue Response in Ovariectomized Rats

Christiane Otto,* Ingrid Kantner,* Reinhard Nubbemeyer, Jenny Schkoldow, Iris Fuchs, Elisabeth Krahl, Richardus Vonk, Christiane Schüler, Karl-Heinrich Fritzemeier, and Reinhold G. Erben

TRG Oncology & Gynecological Therapy (C.O., J.S., E.K., K.-H.F.), Research Pharmacokinetics (R.N.), and Global Drug Discovery Statistics (R.V.), Bayer Pharma AG, 13342 Berlin, Germany; and Department of Biomedical Sciences (I.K., C.S., R.G.E.), University of Veterinary Medicine, Vienna 1210, Austria

Estrogen replacement is an effective therapy of postmenopausal symptoms such as hot flushes, bone loss, and vaginal dryness. Undesired estrogen effects are the stimulation of uterine and mammary gland epithelial cell proliferation as well as hepatic estrogenicity. In this study, we examined the influence of different estradiol release kinetics on tissue responsivity in ovariectomized (OVX) rats. Pulsed release kinetics was achieved by ip or sc administration of estradiol dissolved in physiological saline containing 10% ethanol (EtOH/NaCl) whereas continuous release kinetics was achieved by sc injection of estradiol dissolved in benzylbenzoate/ricinus oil (1/100 vol/vol). Initial 3-d experiments in OVX rats showed that pulsed ip estradiol administration had profoundly reduced stimulatory effects on the uterus and the liver compared with continuous release kinetics. On the other hand, both administration forms prevented severe vaginal atrophy. Based on these results, we compared the effects of pulsed (sc in EtOH/NaCl) vs. continuous (sc in benzylbenzoate/ricinus oil) estradiol release kinetics on bone, uterus, mammary gland, and liver in a 4-month study in OVX rats. Ovariectomy-induced bone loss was prevented by both administration regimes. However, pulsed estradiol resulted in lower uterine weight, reduced induction of hepatic gene expression, and reduced mammary epithelial hyperplasia relative to continuous estradiol exposure. We conclude that organ responsivity is influenced by different hormone release kinetics, a fact that might be exploited to reduce undesired estradiol effects in postmenopausal women. (Endocrinology 153: 1725–1733, 2012)

The female sex hormone estradiol is the clinical gold standard for the treatment of postmenopausal women suffering from vasomotor symptoms and vaginal dryness. Estradiol-treated postmenopausal women still having a uterus require the addition of a progestin to inhibit uterine epithelial cell proliferation and endometrial carcinoma (1). Compared with estradiol-only therapy, combined estrogen-progestin therapy enhances mammary epithelial cell proliferation (2). The Women’s Health Initiative study using conjugated equine estrogens plus medroxyprogesterone acetate reported an enhanced breast cancer risk of the combined hormone therapy arm in comparison with the conjugated equine estrogens-only arm (3). In addition to stimulation of epithelial cell proliferation in the uterus and the mammary gland, hepatic estrogenicity, i.e. induction of clotting factors, angiotensinogen, and sex hormone-binding globulin, is a major side effect of estrogen therapy (4).

The effects of estradiol are mediated by two estrogen receptors, estrogen receptor α (ERα) and β (ERβ) that belong to the superfamily of nuclear hormone receptors (5). In the classical genomic pathway, ligand-bound ER stimulate the transactivation of target genes in the cell nucleus (6). In contrast, nongenomic estradiol effects lead to a rapid activation of cytoplasmic signal transduction cascades, mediated by classical ER associated with the plasma membrane (6).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
Copyright © 2012 by The Endocrine Society
First Published Online February 14, 2012

* C.O. and I.K. contributed equally to this work.

Abbreviations: AUC, Area under the curve; BMD, bone mineral density; B/R, benzylbenzoate/ricinus oil; ER, estrogen receptor; HE, hematoxylin and eosin; OVX, ovariectomized; pQCT, peripheral quantitative computed tomography.
One possible strategy for the development of estrogen therapies with reduced side effects in postmenopausal women is to improve their tissue selectivity, a concept that has been successfully employed in the development of selective ER modulators. Tissue selectivity of estrogens is influenced by the cell-specific expression of ERα and ERβ (5) as well as by the tissue-specific expression of their coactivators and corepressors (7). Therefore, ER isotype-selective ligands or ER ligands stimulating specific coactivator interactions could be used to improve tissue selectivity (8). The availability of ligand and its tissue-specific metabolism is another important parameter. In addition, the selective exploitation of genomic vs. non-genomic estrogen effects may lead to the discovery of novel ER ligands with an improved side effect profile (9). The administration form of estradiol also influences side effects. Whereas oral administration leads to significant hepatic effects, dermal or nasal administration elicits only minor hepatic effects due to a reduced first-pass effect (4). Interestingly, release kinetics of all three administration routes, i.e., oral, nasal, and dermal, are different. Nasal estradiol administration, for example Aerodiol, leads to pulsed release kinetics that are reflected in a rapid estradiol peak within 10–30 min after administration that falls to 10% of its peak levels within 2 h. Compared with nasal estradiol administration, oral or dermal estradiol application resembles more continuous estradiol release kinetics: peak levels of estradiol after oral or dermal administration are reached after 6 and 36 h, respectively, and estradiol levels stay above baseline for 24 h or even longer (10).

Many studies focusing on the efficacy of pulsed vs. continuous estradiol administration were performed in vitro (11, 12). A study in ovariectomized rats reported that pulsed estradiol therapy at very high doses showed less stimulation of uterine epithelial cell proliferation than continuous estradiol release from sc pumps (13). These experiments were complemented by several studies in healthy women. Pulsed estradiol administration (i.e., Aerodiol) was shown to be as efficacious as oral administration with regard to treatment of hot flushes and prevention of bone loss (14, 15). In these studies, all women received an additional progestin to counteract the proliferative activity of estradiol in the uterus (14, 15). Therefore, it was not possible to analyze the estrogen-only effects in the human uterus after pulsed vs. continuous administration. In addition, it was not possible to analyze the distinct effects of pulsed vs. continuous estradiol exposure in human mammary glands. Comprehensive experimental studies examining the effects of pulsed vs. continuous estradiol in different tissues are lacking.

It was the aim of the present study to test the hypothesis that the tissue response to estradiol might be modulated by different release kinetics, and that this strategy could be exploited to improve tissue selectivity of estradiol treatment. For that purpose, we analyzed administration forms mimicking either pulsed or continuous estradiol therapy in ovariectomized rodents and studied the effects of estradiol on vagina, uterus, liver, bone, and mammary gland.

Materials and Methods

Animals and drugs

17β-Estradiol was purchased from Sigma (St. Louis, MO). Female Wistar rats (Charles River Laboratories, Bar Harbor, ME) were maintained in the laboratories of Bayer Pharma AG in Berlin on a 14-h light, 10-h dark cycle and provided with food and water ad libitum. All animal procedures were run according to German animal welfare law and with permission of the District Government of Berlin.

The 4-month experiment in ovariectomized (OVX) rats was performed at the University of Veterinary Medicine in Vienna using Fischer 344 rats (Harlan, Italy) that were maintained on a 12-h light, 12-h dark cycle. Before start of the bone experiments an acclimatization period of 3 months was allowed. Sham-operated (SHAM) rats were fed ad libitum with a standard rat chow (Ssniff R/M-H V1534, Ssniff Spezialdiäten GmbH, Germany). OVX rats were pair fed according to the food intake of the SHAM rats. The study protocol was approved by the Ethical Committee of the University of Veterinary Medicine, Vienna, and by the Austrian Federal Ministry of Science and Research.

Experimental design

Experiment 1: Pharmacokinetics and tissue response in OVX rats treated for 3 d with either pulsed or continuous estradiol

To analyze the effects of pulsed vs. continuous estradiol administration in rats, two different administration forms of estradiol were chosen. To mimic pulsed release, estradiol was dissolved in ethanol/0.15 M NaCl (1 + 9, vol/vol, EtOH/NaCl) and injected ip. For a continuous, slow release, estradiol was dissolved in benzylbenzoate/ricinoleic oil (1 + 4, vol/vol, B/R) and injected sc.

Adult female Wistar rats were OVX. Two weeks after ovariectomy, animals were treated once daily for 3 d with either vehicle or estradiol (0.2, 0.5, 1, and 5 μg/kg body weight; n = 12 animals per treatment group) using the two different ways of administration. For analysis of estradiol levels, blood was taken on d 1 and d 3 at the following time points: 2, 5, 10, 30, 60, 120, 360, and 1440 min after ip administration or at 5, 10, 30, 60, 240, 480, and 1440 min after sc estradiol administration, respectively. Serum was stored at −80°C until analysis.

On d 4, animals were killed and relative uterine weights were determined. Liver samples were frozen in liquid nitrogen for gene expression analysis. The uterine horns and the vagina were fixed in 4% paraformaldehyde in PBS overnight and processed for paraffin embedding. Sections (6-μm-thick) were stained with
Hematolymphoid organs were dissected and weighed. Levels of estradiol in serum were measured by ELISA (DRG Diagnostics, Marburg, Germany) after extraction with diethylether (detection limit is 1.4 ng/liter). Data analysis was performed in Berlin for all data. For pharmacokinetic analysis, geometric mean values were calculated for vehicle groups and for the different treatment groups at each time point. Values obtained from OVX vehicle-treated animals were subtracted from those obtained from estradiol-treated animals. The area under the plasma concentration-time profile from time zero to the last quantifiable time point under the curve (AUC0-tlast) was calculated using linear trapezoidal approximation. The apparent maximum concentration ([Cmax]) and the time at which this concentration was achieved (tmax) were taken directly from the raw data.

**Quantitative RT-PCR**

RNA was isolated after homogenization of livers in guanidinium thiocyanate. RNA (5 μg) was digested with deoxyribonuclease I and reversely transcribed with random hexamers using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time Taqman PCR analysis was performed using the ABI Prism 7700 Sequence Detector System according to the manufacturer’s instructions (PE Applied Biosystems, Foster City, CA). Prevalidated probes and primers for rat achaete-scute complex homolog 1 (Ascl1) and rat Ascl1 (achaete-scute complex homolog 1) (catalog no. Rn00574345-m1) were purchased from PE Applied Biosystems. All experiments were carried out in duplicate. Relative mRNA levels were calculated by the compara-

**TABLE 1. Pharmacokinetic parameters after ip and sc estradiol administration**

<table>
<thead>
<tr>
<th></th>
<th>ip Vehicle: EtOH/NaCl (1 + 9, vol/vol)</th>
<th>sc Vehicle: B/R (1 + 4, vol/vol)</th>
<th>sc Vehicle: EtOH/NaCl (1 + 9, vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (μg/kg/d)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>AUC0-tlast (ng*h/liter)</td>
<td>5.2</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>Cmax (ng/liter)</td>
<td>17.9</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>5</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>tlast (h)</td>
<td>1</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
</tbody>
</table>

n.c., Not calculated because data were not significantly different from baseline values; n.e., not examined.

**Pharmacokinetics**

Pharmacokinetic data for estradiol administered in B/R and EtOH/NaCl ip were obtained in Berlin, whereas pharmacokinetic data for estradiol administered in EtOH/NaCl sc were obtained in Vienna. Estradiol levels at Bayer Pharma AG in Berlin were determined in rat serum as described previously (16) with one modification: diethyl ether extraction of the probes was omitted. The RIA (Beckman Coulter Immunotech, Krefeld, Germany) ranged from 36.7–22,026 pm (10–6,000 ng/liter) estradiol. At the University of Veterinary Medicine in Vienna, serum estradiol was measured by ELISA (DRG Diagnostics, Marburg, Germany) after extraction with diethyl ether (detection limit is 1.4 ng/liter). Data analysis was performed in Berlin for all data. For pharmacokinetic analysis, geometric mean values were calculated for vehicle groups and for the different treatment groups at each time point. Values obtained from OVX vehicle-treated animals were subtracted from those obtained from estradiol-treated animals. The area under the plasma concentration-time profile from time zero to the last quantifiable time point under the curve (AUC0-tlast) was calculated using linear trapezoidal approximation. The apparent maximum concentration ([Cmax]) and the time at which this concentration was achieved (tmax) were taken directly from the raw data.

**Experimental design**

**Experiment 2: Effects of pulsed vs. continuous estradiol in OVX rats treated for 4 months**

Ninety 6-month-old female Fischer 344 rats were randomized according to their body weight into 11 groups. Fifteen animals were sham operated, 75 rats were OVX. Immediately after ovariectomy, animals were treated sc once daily with vehicle or 0.15, 0.5, and 1.5 μg/kg 17β-estradiol dissolved in EtOH/NaCl or in B/R as described above. Vehicle-treated SHAM and OVX rats received both vehicles every day. Because treatment was performed for 4 months, estradiol administration in EtOH/NaCl was performed sc and not ip to keep the animals’ burden as low as possible. Pharmacokinetic analysis was performed in parallel to ensure that sc estradiol injection in EtOH/NaCl mimicked pulsed release kinetics. OVX Fischer rats were sc injected with 1.5 μg/kg estradiol in EtOH/NaCl, and blood samples were obtained by tail vein puncture at 0, 2, 5, 10, 30, 60, 120, 360, and 1440 min after injection. Serum was stored at −80 °C until analysis.

Rats were killed 4 months after ovariectomy by exsanguination from the abdominal aorta under ketamine/xylazine anesthesia (50/10 mg/kg ip). At necropsy, uterine weight was determined. Liver samples were frozen in liquid nitrogen and processed for mRNA analysis as described below. The left tibiae and L4 vertebrae were harvested for analysis of bone mineral density (BMD) using peripheral quantitative computed tomography (pQCT). The left inguinal mammary glands and the uterus horns were fixed in buffered formalin overnight and embedded in paraffin. Sections (7-μm-thick) were prepared and stained with HE. Uterine epithelial cell height was determined as described above.

Pharmacokinetic parameters after ip and sc estradiol administration

<table>
<thead>
<tr>
<th></th>
<th>ip Vehicle: EtOH/NaCl (1 + 9, vol/vol)</th>
<th>sc Vehicle: B/R (1 + 4, vol/vol)</th>
<th>sc Vehicle: EtOH/NaCl (1 + 9, vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (μg/kg/d)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>AUC0-tlast (ng*h/liter)</td>
<td>5.2</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>Cmax (ng/liter)</td>
<td>17.9</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>5</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>tlast (h)</td>
<td>1</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
</tbody>
</table>

n.c., Not calculated because data were not significantly different from baseline values; n.e., not examined.
tive ΔCT method. The expression levels of Ascl1 were normalized to Tbp.

BMD measurements

BMD was measured by pQCT using a XCT Research M+pQCT machine (Stratec Medizintechnik, Pforzheim, Germany). The measurements were made with a collimator opening of 0.2 mm on specimens stored in 70% ETOH. The voxel size was 70 μm. One slice in the mid-diaphysis of the tibia located 2 mm proximal to the tibiofibular junction and one slice in the proximal tibia metaphysis located 2 mm distal from the growth plate were measured. In the L4 vertebra, three slices were measured, one in a mid-transversal plane, and two located 2 mm rostral and caudal of the mid-transversal plane. BMD values of the L4 vertebral body were calculated as mean of three slices. For the measurement of trabecular BMD, we used contour mode 2 for detection of the outer bone edge and peel mode 20 to separate trabecular from cortical-subcortical bone in the L4 vertebra whereas contour mode 1 and peel mode 20 were used in the proximal tibia. The percentage of trabecular bone was set to 50% of the cross-sectional area in the proximal tibia and to 60% of the cross-sectional area in the L4 vertebra. A threshold of 710 mg/cm² was used for calculation of cortical BMD.

Methods for clinical chemistry, bone histology and histomorphometry, and bone biomechanics are described in the Supplemental data published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org

Statistical analysis

Wilcoxon rank-sum tests were used to assess differences between the application forms for the following readout parameters: relative uterine weight, uterine epithelial cell height, and Ascl1 induction. To assess for multiplicity, for all parameters a stepwise sequential rejection method (using an α-level of 5%) was used to control the family-wise error rate.

For the 4-month experiment in OVX rats, statistics were computed using SPSS for Windows 17.0. Data were analyzed by one-way ANOVA. When the ANOVA performed over all groups indicated a significant difference among groups, statistical differences between individual groups were subsequently evaluated using Student-Newman-Keuls’s multiple comparison test as post hoc test. P < 0.05 was considered significant. Data are presented as mean ± SD.

Results

Pharmacokinetics of estradiol administered in EtOH/NaCl or B/R

To assess the release kinetics of pulsed vs. continuous estradiol treatment, we injected OVX rats ip or sc for 3 consecutive days with estradiol dissolved in EtOH/NaCl or in B/R, respectively. On the first and third day, estradiol serum levels were determined at different time points after drug administration. No differences in estradiol serum concentrations were observed between d 1 and d 3. Values obtained from OVX vehicle-treated animals were subtracted from the data of the treatment groups. Pharamacokinetic analyses are depicted in Table 1. After ip administration, estradiol serum concentrations peaked early at 2–5 min (t_max). C_max increased with higher doses. The exposure (AUC_{0–t}) increased in a dose-dependent manner (except the 1.0 μg dose group). Subcutaneous estradiol administration in B/R resulted in much lower peak concentrations (C_max) at later time points (t_max) compared with ip administration. No elevation of estradiol levels above baseline was observed after sc administration of 0.2 and 0.5 μg/kg estradiol. This finding was in line with previous reports showing no elevation of basal estradiol levels after sc administration of 0.25 μg/kg estradiol in B/R (16).

In general, no elevation of serum estradiol levels is detectable in OVX rats after sc administration of estradiol doses <1 μg/kg (E. Krahl, personal communication). Therefore, it was only possible to calculate the AUC_{0–t} after dosing of 1 and 5 μg/kg estradiol in B/R sc (Table 1). The AUC_{0–t} vs. 

![Image](https://academic.oup.com/endo/article-abstract/153/4/1725/2423866)

**FIG. 1.** Uterine and hepatic effects in rats after pulsed vs. continuous administration of estradiol for 3 d in rats. Two weeks after ovariectomy, rats were treated for 3 d with either vehicle or the indicated estradiol doses either ip in EtOH/NaCl (solid circles and bars) or sc in B/R (open circles and bars). Necropsy was performed on d 4. Relative uterine weight (A) and uterine epithelial cell height (B) increased to a greater extent after sc compared with ip estradiol administration. In the liver, the estrogen target gene Ascl1 was more responsive to sc than to ip estradiol administration. Data are presented as mean ± SD; asterisks indicate significant differences between identical estradiol doses after ip and sc administration (*, P < 0.05).
In these two groups showed a dose proportional increase. Intraperitoneal estradiol administration in EtOH/NaCl provoked short lasting, rather high peak levels, reflecting a pulsed release pattern, whereas sc application of the same doses in B/R resulted in much lower peak levels, that were maintained over a longer period of time reflecting a more continuous drug release. The pharmacokinetic analysis of estradiol administration in EtOH/NaCl sc as it was used in the 4-month study in OVX rats showed a more protracted release pattern compared with ip administration in the same vehicle (Table 1). However, compared with the relative slow release and low peak levels after sc administration in B/R, sc administration in EtOH/NaCl still can be considered as a pulsed release pattern.

**Pulsed estradiol has reduced uterine and hepatic effects compared with continuous estradiol after 3 d of treatment**

In the same OVX animals that were used for pharmacokinetic analysis we determined relative uterine weight, uterine epithelial cell height, and liver gene induction after 3 d of sc (B/R) or ip (EtOH/NaCl) estradiol treatment. Intraperitoneal estradiol administration stimulated relative uterine weight (Fig. 1A) and epithelial cell height (Fig. 1B) to a lower extent if compared with sc estradiol administration. To monitor hepatic estradiol effects, we analyzed the expression of Ascl1 (achaete-scute complex homolog 1), a hepatic gene that is induced by estradiol in a dose-dependent manner (C. Otto, unpublished observation). Subcutaneous estradiol administration induced Ascl1 expression more efficiently than ip administration (Fig. 1C).

Ovariectomy caused severe atrophy of the vaginal epithelium (Fig. 2, A and F). Full estrogenic activity in the rat vagina is indicated by the presence of a cornified epithelium (asterisks). Intraperitoneal estradiol administration showed full estrogenic activity at 5 μg/kg whereas sc administration exhibited full estrogenic activity from 0.5 μg/kg onward. Notably, ip administration of 0.2 μg/kg estradiol already prevented severe vaginal atrophy but the vaginal epithelium was thinner compared with the effects of the same estradiol dose given sc in B/R (G).

![Figure 2](https://academic.oup.com/endo/article-abstract/153/4/1725/2423866)

**FIG. 2.** Effects of pulsed and continuous estradiol (E2) on vaginal epithelium in OVX rats. Two weeks after ovariectomy, rats were treated for 3 d with either vehicle (A and F) or the indicated estradiol doses either ip in EtOH/NaCl (B–E) or sc in B/R (G–J). Necropsy was performed on d 4. Photographs of HE-stained vaginal sections were taken at the same magnification. Full estrogenic activity was indicated by the presence of a cornified epithelium (asterisks). Intraperitoneal estradiol administration showed full estrogenic activity at 5 μg/kg whereas sc administration exhibited full estrogenic activity from 0.5 μg/kg onward. Notably, ip administration of 0.2 μg/kg estradiol already prevented severe vaginal atrophy but the vaginal epithelium was thinner compared with the effects of the same estradiol dose given sc in B/R (G).

Similar effects on bone, but reduced effects on liver, uterus, and mammary gland after 4-month treatment of OVX rats with pulsed vs. continuous estradiol

To examine the long-term effects of pulsed vs. continuous estradiol on bone and other organs, we performed a 4-month experiment in 6-month-old OVX Fischer rats. As mentioned above, to reduce the animals’ burden we administered estradiol dissolved in EtOH/NaCl sc instead of ip to mimic a pulsed estradiol release pattern.

As expected, ovariectomy induced uterine atrophy (Fig. 3, A and B) and high turnover osteopenia as evidenced by reduced total and trabecular proximal tibial and lumbar vertebral BMD (Fig. 3, C–F). In addition, ovariectomy increased biochemical markers of bone formation and bone resorption (Supplemental Fig. 1). Estradiol treatment of OVX rats increased uterine weight (Fig. 3A) and epithelial cell height (Fig. 3B) in a dose-dependent manner. As observed in the short-term rat study, estradiol administered in B/R increased uterine weight and epithelial cell
height to a greater extent than estradiol administered in EtOH/NaCl at doses of 0.5 and 1.5 μg/kg (Fig. 3, A and B). However, estradiol given in both vehicles protected against ovariectomy-induced loss of tibial and vertebral BMD (Fig. 3, C–F) and against ovariectomy-induced loss of bone strength (Supplemental Fig. 1) in a dose-dependent manner by suppressing bone turnover. Serum alkaline phosphatase, serum osteocalcin, urinary deoxypyridinoline excretion, and vertebral cancellous bone formation rate were suppressed in a dose-dependent manner to the same extent by estradiol treatment in both vehicles (Supplemental Fig. 1).

As observed in the short-term experiments, pulsed estradiol administration stimulated hepatic Ascl1 expression to a reduced extent compared with estradiol administered in B/R (Fig. 4). HE-stained mammary gland sections are depicted in Fig. 5. Mammary gland epithelium in OVX rats (Fig. 5B) appeared atrophic when compared with SHAM animals (Fig. 5A). Increasing doses of estradiol administered in B/R (Fig. 5, C, E, and G) and in EtOH/NaCl (Fig. 5, D, F, and H) stimulated epithelial cell hyperplasia. A dose of 1.5 μg/kg estradiol applied in EtOH/NaCl (Fig. 5H) induced ductal growth and epithelial proliferation in a similar fashion as in SHAM animals (Fig. 5A). In sharp contrast, administration of 1.5 μg/kg estradiol in B/R (Fig. 5G) provoked mammary gland hyperplasia as well as secretory activity that clearly exceeded the effects seen in SHAM animals (Fig. 5A).

Taken together, the results obtained from two independent experiments in rats demonstrated that pulsed estradiol release, i.e., application of estradiol in EtOH/NaCl either sc or ip, provoked less undesirable effects in the uterus, the liver, and the mammary gland compared with continuous estradiol release after sc application of estradiol in B/R. On the other hand, pulsed estradiol release successfully prevented ovariectomy-induced bone loss and vaginal atrophy.

**Discussion**

The focus of our present work was to examine whether different estradiol release kinetics may influence hormone responsivity in different target tissues. To answer this question, we established hormone release conditions mim-
Data are presented as mean ± SD; asterisks indicate significant differences between identical estradiol doses after ip and sc administration (*, P < 0.05).

The findings made in the current study are in line with clinical studies investigating the efficacy of nasal estradiol administration, leading to pulsed estradiol exposure (14, 15). Postmenopausal women receiving the nasal estradiol spray Aerodiol were efficiently treated against hot flushes and bone loss but showed less mastalgia and withdrawal bleeding, suggesting reduced activity in the mammmary gland and uterus when compared with women receiving estradiol orally, which results in a more continuous estradiol release (10). Because women were cotreated with a progestin, the analysis of estradiol-only effects in the uterus and the mammary gland was not possible in these studies. Nevertheless, our results obtained in rodents corrobate the notion that administration of pulsed estradiol leads to reduced activity in the uterus and mammary gland.

Although caution must always be used when extrapolating findings from rodents to humans, our findings may suggest that pulsed (e.g. nasal) estradiol administration in postmenopausal women may require the addition of lower progestin doses to inhibit proliferation in the uterus compared with continuous (e.g. oral) estradiol application which stimulates uterine epithelial cell proliferation to a greater extent. Preclinical studies (18) suggested a direct dose-response relationship between mammary gland epithelial cell proliferation and applied progestin dose at a fixed estradiol dose. Thus, a possible additional benefit of pulsed estradiol in nonhysterectomized women taking combined hormone therapy may be a reduced proliferative activity in the breast due to lower progestin doses required.

With respect to the uterine effects, our results are in accordance with an earlier study performed in rats (13), where estradiol was administered either iv to mimic pulsed release of estradiol or sc with an osmotic minipump to mimic continuous release. Although estradiol dosing was much higher, up to 250 μg/kg body weight per d, the observations made in the uterus were similar to our study. Relative uterine weight, epithelial cell height, and epithelial cell proliferation were lower after pulsed compared with continuous administration (13). Previously, it was suggested that pulsed estradiol administration may lead to higher activation of nongenomic estradiol signaling pathways than continuous exposure (19), which, in turn, might change ERα and ERβ expression patterns in the uterus and may lead to decreased epithelial cell proliferation after pulsed administration (13). Contrary to this latter suggestion, it was shown recently that estrogenic effects in the mammary gland, in the uterus, and in the liver were completely blunted in mice that expressed a mutant ERα that was not able to bind to DNA but still activated nongenomic responses (20). We therefore hypothesize that the genomic pathway activated by the classical ERα is responsible for undesired as well as for the desired effects of hormone therapy.

Our pharmacokinetic data suggest that the observed target organ effects were not peak driven. Otherwise there should have been stronger uterine effects after ip and sc estradiol administration in EtOH/NaCl (producing much higher estradiol peak levels) than after sc administration in B/R. This was clearly not the case. Instead, we observed the opposite behavior. Because it was not possible to reliably determine estradiol levels after sc application of doses lower than 1 μg/kg (16), we could not calculate AUC and
total estradiol exposure for the lower estradiol doses in B/R. Therefore, it remains an open question whether the biological response was AUC driven or determined by the maintenance of estradiol levels above a defined threshold for a certain period of time. In vitro it was shown that at the same exposure (AUC), endothelial nitric oxide synthase was induced to the same extent after pulsed and continuous estradiol stimulation in human umbilical vein endothelial cells (12). In vivo, different estradiol target organs might exhibit distinct responsivity to pulsed or continuous estradiol due to differences in ER density, coactivator expression, or intracellular metabolism of estradiol (5, 7).

Collectively, the clinical studies comparing nasal and oral estradiol administration (14, 15) as well as our study and the other study performed in rats (13) using pulsed and continuous estradiol release in rodents, suggest that the central nervous system, the bone, and the vaginal epithelium seem to be more responsive to pulsed estradiol than the uterus, the mammary gland, and the liver. The latter organs appear to require a more sustained stimulation for full estrogenic efficacy. The available clinical and experimental data suggest that pulsed estradiol release may be used in postmenopausal hormone therapy to efficaciously treat hot flushes, bone loss, and vaginal dryness. Compared with oral hormone therapy and patches, pulsed estradiol release, as for example achieved with nasal administration, may reduce unwanted side effects in the uterus, the mammary gland, and the liver in hormone therapy. Currently, the molecular mechanisms underlying the different tissue responsivity toward different estradiol release kinetics are unknown. Elucidation of these mechanisms may open up new perspectives for the development of estrogen therapies with an improved risk-benefit ratio.

**Acknowledgments**

We thank Verena Spielberger, Kerstin Klien, and Claudia Bergow (Department of Biomedical Sciences, University of Veterinary Medicine, Vienna 1210, Austria) for excellent technical assistance.
Address all correspondence and requests for reprints to: Christiane Otto; TRG Oncology & Gynecological Therapy, Bayer Pharma AG, 13342 Berlin, E-mail: christiane.otto@bayer.com.

Disclosure Summary: C.O., R.N., J.S., I.F., E.K., R.V., and K.H.F. are employed by Bayer Pharma AG in Berlin; R.G.E. has received research grants from Bayer Pharma AG, and I.K. and C.S. have nothing to declare.

References

2. Hofseth LJ, Raafat AM, Osuch JR, Pathak DR, Slomski CA, Haslam SZ 1999 Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. J Clin Endocrinol Metab 84:4559–4565