Estrogen receptor subtypes selectively mediate female mouse reproductive abnormalities induced by neonatal exposure to estrogenic chemicals

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1. Introduction

Estrogens tightly regulate cell proliferation and differentiation particularly in the oviduct, uterus, vagina and mammary gland of the female reproductive tracts. Long-term estrogenic stimulation is known to be a risk factor for carcinogenesis in humans and laboratory animals (Marselos and Tomatis, 1992a,b). Transplacental exposure to synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for pre-conceptional exposure to the synthetic estrogen, diethylstilbestrol (DES) and to estrogenic chemicals, induces persistent anovulation caused by alteration of hypothalamic–pituitary–gonadal (HPG) axis, polyovular follicles, uterine abnormalities and persistent vaginal changes in mice. Most activities of estrogenic chemicals are mediated through estrogen receptor α (ERα) and/or ERβ. However, little was known about the relative contribution of the individual ER subtypes in induction of abnormalities. We tested the effects of neonatal exposure to ER selective ligands and DES on female mice. Transactivation assays using mouse ERs and ERβ showed that 10−10 M DES activated both ER subtypes and that the ERα agonist (propyl pyrazole triol, PPT) and the ERβ agonist (diarylpropionitrile, DPN) selectively activated their respective ERs at 10−8 M. Neonatal female mice were injected subcutaneously with DES, PPT or DPN and the animals were examined at 13 and 15 weeks of age, respectively. Persistent estrous smears and anovulation were induced in all mice by 0.025–2.5 µg DES and 2.5–25 µg PPT, but not by DPN, suggesting that the observed anovulation was primarily mediated through ERα. Disorganization of uterine musculature and ovary-independent vaginal epithelial cell proliferation accompanied by persistent expression of EGF-related genes and interleukin-1-related genes were also mediated through ERα. In contrast, polyovular follicles were induced by neonatal treatment with both ERα and ERβ ligands, suggesting that ovarian abnormalities are mediated through both ER subtypes.

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Estrogens act primarily through the nuclear estrogen receptors, ERα and ERβ, in mammals. ERα and ERβ can be detected in a broad spectrum of tissues. In some organs, both ER subtypes are expressed at similar levels, whereas in others, ERα or ERβ predominate. In addition, both ER subtypes may be present in the same tissue, but in different cell types. ERα is mainly expressed in the uterus, prostate (stroma), ovary (theca cells), testis (Leydig cells), epididymis, bone, breast, various regions of the brain, liver and white adipose tissue. ERβ is expressed in colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelial cells and certain regions of the brain (Weihua et al., 2003; Dahlman-Wright et al., 2006).

ERα knockout (αERKO) mice were used to study the action of DES. In wild-type mice, uterine expression of the genes Hoxa10, Hoxa11 and Wnt7a exhibited significant decreases shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). An expression study of αERKO mice provided unequivocal evidence supporting an obligatory role for ERα in mediating the detrimental actions of neonatal DES exposure in the murine reproductive tract (Couse et al., 2003) reported that ERα, but not ERβ, is indispensable in the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary. ERα appears to be the predominant ER in the adult mouse uterus, vagina, oviduct and mammary gland (Couse et al., 2000; Korach et al., 2003).

Immunohistochemical localization of ERβ was demonstrated only in differentiating granulosa cells of the ovary where ERα was observed prominently in interstitial cells. ERα mRNA was expressed in the female reproductive tract at all ages examined with little or no significant levels of ERβ, except on postnatal day 1 when a low level of message appeared (Jefferson et al., 2000). ERβ was detectable in the uterus of both wild-type and αERKO mice, but only at very low levels (Korach et al., 2003). The significance of ERβ in the induction of polyovular follicles by genistein in mice has been reported by Jefferson et al. (2002). Bodo et al. (2006) demonstrated that both ERα and ERβ are involved in the sexual differentiation of the anteroventral periventricular area in the mouse hypothalamus.

On the other hand, estrogenic chemicals in the environment have been shown to have potential adverse effects on animals and humans exposed during embryonic developmental stage (Damstra et al., 2002). Most of estrogenic chemicals bind to ERα better than ERβ, but some chemicals bind to ERβ better than ERα (Kuiper et al., 1997). Thus, importance of ER subtypes needs to be studied in induction of adverse effect by estrogenic chemicals.

Recently, ERα- and ERβ-specific ligands have been synthesized and characterized using transactivation assays (Harris et al., 2002; Frasier et al., 2003; Katzenellenbogen et al., 2003). In this study, we investigated the expression of each ER subtype in the induction of anovulation through the hypothalamic–pituitary axis with persistent estrus, permanent vaginal epithelial cell proliferation, disorganization of uterus, and in the induction of polyovuliferous follicles in mice exposed neonatally to selective ER ligands or DES.

2. Materials and methods

2.1. Reagents

Diethylstilbestrol (DES), 17β-estradiol (E2) and ethynylestradiol (EE2) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4′-′(4-propyl-1H)-pyrazole-1,3,5-triyl[risphenol (propyl pyra-

zole triol, PPT) and ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Estrogen receptor transactivation assay

CHO-K1 cells were seeded in 24-well plates at 5 × 10^4 cells/well in phenol-

red free Dulbecco’s modified Eagle’s medium (Sigma–Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA). The cells were transfected overnight with 400 ng of pGL3-

Basic-4-ERE-tk-luc, 100 ng of pRL-tk-luc (as an internal control to normalize for variation in transfection efficiency); contains the Renilla reniformis luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega, Madison, WI, USA), and 200 ng of pTARGET-mERα (mERα) or mERβ using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer-recommended protocol.

After 18 h, doses ranging from 10^-12 to 10^-6 M of DES, PPT, DPN, E2 or EE2 were administered to the culture media. Twenty-four hours after the cultures treated with ligands, the cells were collected and the luciferase activities of the cells were measured using a chemiluminescence assay with Dual-Luciferase Reporter Assay Sys-

tem (Promega). Luminescence was measured using a Turner Designs Lumimeter TD-20/20 (Promega). Promoter activity was calculated as firefly (Photinus pyralis)–luciferase activity/sea pansy (R. reniformis)–luciferase activity (Katsu et al., 2006). All transfection assays were repeated 5 times.

2.3. Animals and treatments

Female C57BL/6j mice were maintained under 12 h light/12 h dark at 23–25 °C and fed laboratory chow (CE-2, CLEA, Tokyo, Japan) and tap water ad libitum. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

Three female newborn mice were sacrificed and the hypothalamus, ovary, uterus and vagina were dissected to measure expression levels of ERα and ERβ mRNA. The other female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025, 0.25 or 2.5 μg DES/g body weight (BW) dissolved in sesame oil, 0.25, 2.5 or 25 μg/g BW PPT or DPN, dissolved in 5.6% DMSO or the vehicle alone beginning from day 0 (the day of birth). These mice were ovariopectomized at 13 weeks and sacrificed at 15 weeks of age.

Vaginal smears were recorded from 11 weeks of age for 4 weeks. After ovariec-
tomy, the dissected ovaries were weighed and fixed in 10% neutral buffered formalin. At 15 weeks of age, 6 mice in each experimental group treated with the highest con-

centrations of DES, PPT and DPN, and oil controls were given a single injection of 50 μg of Brdu/μg BW 2 h before sacrifice. The vagina was cut in half longitudinally and one horn of each uterus was weighed. Half of the tissue was fixed in 10% neutral buffered formalin and the other half was frozen in liquid nitrogen for the analysis of gene expression.

In addition, 8–19 newborn female mice were given 5 daily injections of 2.5 μg DES/g BW, 25 μg PPT or DPN/g BW or the vehicle alone. These mice were used for analysis of polyovular follicles in the ovary at 30 days of age.

2.4. Hematoxylin and Eosin (HE) staining and Brdu immunostaining

Tissues were embedded in paraffin, sectioned at 8 μm, following by standard HE staining and analysis of the ovaries, uterus and vaginae. Parts of deparaffinized sections were incubated in 0.3% H2O2 in methanol for 30 min, then immersed in 2N HCl for 20 min in order to denature the genomic DNA. After washing with PBS (PBS in 0.5% Tween), the sections were incubated with anti-Brdu antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1:20 in PBS containing 1% BSA overnight at 4 °C.

The sections were subsequently incubated with 3,3-diaminobenzidine tetra-
dichloride containing hydrogen peroxide. Brdu-labeling index was estimated by counting the number of Brdu-incorporated cells per h in the basal layer of cells in the vaginal epithelium as described previously (Miyagawa et al., 2004a,b). Polyovuliferous follicles containing more than one oocyte in a follicle bigger than 50 μm were histologically examined and counted as described previously (Iguchi et al., 1998).

2.5. Real-time quantitative RT-PCR

Changes in gene expression were confirmed and quantified using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, USA). Total RNA,
isolated with RNEasy kit (QIAGEN, Chatsworth, CA, USA) from each group, was used in RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer’s instructions. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 36 cycles of 95°C for 15 sec and 60°C for 1 min in 15 μl volumes. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 1. More than three pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.

2.6. Statistical analysis

Statistical analysis was performed using one-way or two-way analysis of variance, Fisher’s exact probability test, Student’s t-test or Welch’s t-test followed by F-test as appropriate. Differences with P < 0.05 were considered significant.

3. Results

3.1. Estrogen receptor transactivation assay

Transactivation assays with ERα revealed that DES, E2 and EE2 showed high activity at 10⁻¹⁰ M, whereas the ERα-specific ligand, PPT, showed the highest transactivation activity at 10⁻⁹ M. The estrogenic activity of PPT toward mERα was 10 times less than DES, E2 and EE2. The ERβ-specific ligand, DPN, showed no significant estrogenic activity to mERα, confirming that PPT is an ERα specific ligand (Fig. 1A).

Transactivation of mERβ showed the highest activity of DES at 10⁻¹⁰ M, and E2 and EE2 at 10⁻⁹ M. DPN, showed highest transactivation activity at 10⁻⁹ M. As with PPT and ERα, the estrogenic activity of DPN toward mERβ was 10 times less than DES, E2 and EE2. PPT showed no significant estrogenic activity toward mERβ, confirming that DPN is an ERβ specific ligand (Fig. 1B). Based on these results, the doses of PPT and DPN for neonatal mouse were set 10 times higher than that of DES.

3.2. Vaginal smear observation

Vaginal smears were observed daily from 11 to 15 weeks of age (from 2 weeks before ovariectomy until 2 weeks after ovariectomy). Control mice showed regular estrous cycles before ovariectomy and diestrous type smears after the ovariectomy. All mice given 0.025–2.5 μg DES showed constant estrous smears before ovariectomy and 4 of 11 mice exposed to 0.025 μg, and all of 0.25 and 2.5 μg DES exposed mice showed constant estrous smears even after ovariectomy. Seven of 11 mice exposed to 0.025 μg DES showed diestrous type smears after ovariectomy. Three of 10, 10 of 11 and 16 mice treated neonatally with 0.025, 0.25 and 2.5 μg PPT, respectively, showed constant estrous smears even after ovariectomy. The remaining 7 of 10 and 1 of 11 mice treated with 0.25 and 2.5 μg PPT, respectively, showed estrous cycles before ovariectomy. After ovariectomy, 9 of 10 and 9 of 11 mice at the 0.25 and 2.5 μg doses of PPT showed diestrous smears after ovariectomy. The remaining 1, 2 and 16 mice treated with 0.25, 2.5 and 25 μg PPT, respectively, showed persistent estrous smears even after ovariectomy. In neonatally DPN treated mice, 10 of 10 mice at the 0.25 μg dose, and 5 of 16 at the 25 μg dose showed constant estrous smears. The remaining mice showed regular estrous cycles before ovariectomy. After ovariectomy, 2 of 16 mice at the 25 μg DPN dose showed persistent estrous smears, the rest showed diestrous smears.

3.3. Ovarian histology

Ovaries dissected at 13 weeks of age were examined histologically. All control mice showed corpora lutea in the ovary indicating that ovulation had occurred. However, all DES-exposed mice lacked corpora lutea in the ovary, demonstrating anovulation even at the lowest (0.025 μg/g BW) concentration. A significantly higher incidence of anovulation was found in mice exposed neonatally to 2.5 and 25 μg PPT, however, no significant increase in the num-

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### Table 1

Sequences of gene primer sets for real-time quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
<th>Product size (bp)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arfg</td>
<td>F: CATTATGAGGTGCCCTTTTGA&lt;br&gt;R: TTTGCCATTATGGTGGAAAAC</td>
<td>124</td>
<td>NM_009704</td>
</tr>
<tr>
<td>Erfg</td>
<td>F: CGCTGTCCTTGCCTAGGC&lt;br&gt;R: GGATCTGCCCTCGATGGA</td>
<td>122</td>
<td>NM_007950</td>
</tr>
<tr>
<td>Hbegf</td>
<td>F: GACACATGGGCGCAGAATA&lt;br&gt;R: GGCATTCCAAGAGGGAGTA</td>
<td>89</td>
<td>NM_010415</td>
</tr>
<tr>
<td>Ilk2</td>
<td>F: GTGTATCTTCCTGCTTACCAC&lt;br&gt;R: CAAAATACGCCACATCTCCAC</td>
<td>101</td>
<td>NM_010555</td>
</tr>
<tr>
<td>nip</td>
<td>F: GGCACCAAGAATCAAAGAC&lt;br&gt;R: CGCATCCAGAAGGAGGTA</td>
<td>69</td>
<td>NM_019451</td>
</tr>
<tr>
<td>Esrl</td>
<td>F: AATGAAATGCGTCTCGG&lt;br&gt;R: AAAGCCAAGGCGGGTATT</td>
<td>98</td>
<td>NM_007956</td>
</tr>
<tr>
<td>Esr2</td>
<td>F: CTACAGTGTCCCAGCAGCA&lt;br&gt;R: GCATAGAACAGCGTACAGGG</td>
<td>136</td>
<td>NM_010157</td>
</tr>
</tbody>
</table>

Areg (amphiregulin), Erfg (epiregulin), Hbegf (heparin binding-epidermal growth factor), Ilk2 (interleukin-1 receptor type II), Hip (IL-1 family, member 5), Esr1 (estrogen receptor α, ERα) and Esr2 (ERβ).

* F, forward; R, reverse.
Table 2

Effects of neonatal exposure of ERα- and ERβ-ligands on ovary (13 weeks), uterus and vagina (15 weeks) in mice.

<table>
<thead>
<tr>
<th>Treatments (μg/g BW)</th>
<th>No. of mice used</th>
<th>No. of mice with</th>
<th>Ovary without corpora lutea</th>
<th>Uterine muscle disorganization</th>
<th>Vaginal epithelial stratification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.025 DES</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25 DES</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>1*</td>
</tr>
<tr>
<td>2.5 DES</td>
<td>14</td>
<td>14*</td>
<td>14*</td>
<td>14*</td>
<td>14*</td>
</tr>
<tr>
<td>0.25 PPT</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2.5 PPT</td>
<td>11</td>
<td>10*</td>
<td>9*</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>25 PPT</td>
<td>16</td>
<td>16*</td>
<td>16*</td>
<td>16*</td>
<td>16*</td>
</tr>
<tr>
<td>0.25 DPN</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5 DPN</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25 DPN</td>
<td>16</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*P < 0.05 vs controls (Fisher’s exact probability test).

The number of mice showing anovulation was induced by 0.25 μg of PPT or by any dose of DPN exposure (Table 2 and Fig. 2). Hyperplastic interstitial cells and lack of corpora lutea were encountered in the ovaries of mice exposed neonatally to DES (0.025–2.5 μg) and 2.5 and 25 μg PPT. In all treatment groups, mice showed regular estrous cycles before ovariecomy had corpora lutea, whereas, mice showing constant estrous smears lacked corpora lutea in the ovary.

3.4. Uterine and vaginal histology

The uterine epithelium was composed of a single layer of low columnar cells with several uterine glands and circular and longitudinal muscle layers in ovariectomized control and 0.25–25 μg DPN-exposed mice. Disorganization of stromal cells and muscle layers, such as hypoplasia of circular muscle and decrease in density of longitudinal muscle, was encountered in mice treated neonatally with 0.025–2.5 μg DES and 2.5 and 25 μg PPT (Table 2 and Fig. 2). The vaginal epithelium of neonatally oil-injected, 15-week-old ovariectomized control and 0.25–25 μg DPN-exposed mice was composed of 2–3 layers of cuboidal cells. The vaginal epithelium of the age-matched, neonatally 0.25–2.5 μg DES- and 25 μg PPT-exposed, ovariectomized mice exhibited stratification and superficial keratinization (Table 2 and Fig. 3). In the vagina showing ovary-independent epithelial stratification, the basal cells in the epithelium showed high proliferative activity (18–19%), which was confirmed by BrdU immunostaining. In contrast, the basal cells in the vaginal epithelium of control and DPN-treated ovariectomized animals showed very low incidence (1.7%) of BrdU incorporation (Fig. 3E).

3.5. Persistent expression of growth factor and IL-1-related genes

mRNA expression of Areg, Ereg, Hbegf, Il1r2, Il1f5, ERα and ERβ in the vagina was analyzed using real-time quantitative RT-PCR in mice exposed neonatally to 2.5 μg DES, 25 μg PPT and 25 μg DPN. The vaginae of mice exposed neonatally to DES and PPT showed persistent expression of these genes, but not the vaginae of mice exposed to oil or to DPN (Fig. 3F). In addition, the expression of ERα mRNA in the vagina of ovariectomized control mice were 1000 times higher than that of ERβ (data not shown), exhibiting that ERα is the predominant estrogen receptor in the vagina. A piece of vagina used for mRNA analysis was also histologically analyzed. We confirmed the epithelial stratification in neonatally DES- and PPT-exposed mice, but not in controls and mice exposed neonatally to DPN.

3.6. Induction of polyovular follicles

Ovaries dissected at 30 days of age were histologically examined. A high incidence of polyovular follicles (PFs) was found in ovaries of mice exposed neonatally to 25 μg PPT and DPN (3.1% and 4.3%, respectively), although, no significant difference was found...
in the incidence of PFs between mice exposed to PPT or DPN. Mice exposed to 2.5 μg DES exhibited the highest incidence of PFs in the ovary (14%), showing that DES is the most potent inducer of PFs among chemicals used in this experiment (Table 3 and Fig. 4).

### Table 3

Incidence of polyovular follicles (PFs) in 30-day-old mice treated neonatally with ERα- and ERβ-ligands.

<table>
<thead>
<tr>
<th>Treatments (μg/g BW)</th>
<th>No. of mice examined</th>
<th>PFs frequency (No. of mice with PFs)a</th>
<th>Incidence of PFs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>13</td>
<td>77 (10)</td>
<td>0.6 ± 0.14b (2)c</td>
</tr>
<tr>
<td>DES (2.5)</td>
<td>8</td>
<td>100 (8)</td>
<td>14.0 ± 0.84 (2–7)</td>
</tr>
<tr>
<td>PPT (25)</td>
<td>15</td>
<td>100 (15)</td>
<td>3.1 ± 0.43 (2–6)</td>
</tr>
<tr>
<td>DPN (25)</td>
<td>19</td>
<td>100 (19)</td>
<td>4.3 ± 0.58 (2–3)</td>
</tr>
</tbody>
</table>

a Ratio of mice with PFs (%).
b Mean ± S.E.
c Range of the number of oocytes/PF in parentheses.

*P < 0.05 vs controls (one-way ANOVA).

3.7. Expression of ERα and ERβ in various tissues of female newborn mice

The ratio of ERα to ERβ mRNA in the hypothalamus, ovary, uterus, and vagina were analyzed using real-time quantitative RT-PCR in newborn female mice. ERα mRNA expression was higher than that of ERβ mRNA in all tissues examined: hypothalamus, 15.0 ± 2.4; ovary, 17.8 ± 4.9; uterus, 33.7 ± 6.1 and vagina, 27.7 ± 5.8 (the value indicates the ratio of ERα mRNA to ERβ mRNA, mean ± S.E.), demonstrating that ERα is the predominant estrogen receptor in these tissues in female newborn mice.

### 4. Discussion

We confirmed the selective activation of ER subtypes reviewed previously by Katzenellenbogen et al. (2003) using transactivation assays with mouse ERα and ERβ (Katsu et al., 2006). In the ERα
assay, E2 and DES maximally activated the reporter gene at $10^{-10}$ M and PPT activated it at $10^{-9}$ M. In the ERβ transactivation assay, E2, DES and DPN maximally activated the reporter gene at $10^{-10}$ M. Based on these data, the dose of PPT and DPN to be used for in vivo studies was set 10 times higher than DES.

In rodents, administration of aromatizable androgen or estrogen to neonatal females induces anovulatory sterility (Barraclough, 1961; Takewaki, 1962; Gorski, 1963; Takasugi, 1976; Iguchi et al., 1988; Aihara and Hayashi, 1989; Kincl, 1990; Iguchi, 1992), whereas castration of neonatal male rats evokes the capacity for sexual cyclicity and lordosis behavior that is characteristic of the female rat. These treatments are effective only during the “critical period” of perinatal life, and the steroids given are considered to masculinize or defeminize the brain (Goy and McEwen, 1980; Iguchi et al., 1988). ERα and ERβ expression has been demonstrated in the mouse brain (Mitra et al., 2003). ERα knockout (αERKO) mice are sterile. αERKO mice are fertile, but the average number of offspring is less than for wild-type mice (Couse and Korach, 1999; Couse et al., 2003). This demonstrates the critical importance of ERα in the normal development of the hypothalamic–pituitary–ovarian (HPG) axis. Couse et al. (2003) further demonstrated that ERα is indispensable to the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary.

Plastic component, bisphenol-A (BPA) and phytoestrogen genistein bind to ERβ about 7 times better than they do ERα (Kuiper et al., 1997). Neonatal exposure to BPA induced anovulatory sterility in female rats (Kato et al., 2003). Also, neonatal exposure to BPA, or to genistein affected sexual differentiation of the anteroven-tral periventricular nucleus of the hypothalamus (Patisaul et al., 2006). We, therefore, studied effects of neonatal exposure to ER selective ligands on the hypothalamus. In the present study, vaginal smears of mice exposed neonatally to 0.025–2.5 μg DES and to 2.5 and 25 μg of the ERα specific ligand, PPT, showed persistent estrus. However, mice exposed to the ERβ specific ligand, DPN, showed cyclic smear patterns. Mice showing persistent estrous smear patterns that were exposed neonatally to DES and PPT had no corpus luteum in the ovary, indicating anovulatory sterility. These results clearly suggest that ERα, but not ERβ, mediates most of the estrogenic effects of chemicals on the HPG axis during critical developmental stages.

In newborn mice, ERα is localized in the uterine stromal, but not in the epithelial cells whereas it is expressed in both epithelial and stromal cells in the vagina (Sato et al., 1992). The present study confirmed that ERα is the predominant form of ER in the uterus and vagina as reported previously (Jefferson et al., 2000; Couse and Korach, 2004). The present study demonstrated that the ratio of ERα/ERβ is bigger in the adult vagina than the newborn vagina.

In tissue recombination experiments, ERα-negative uterine epithelium (derived from the αERKO mouse uterus) recombined with ERα-positive stroma, showed proliferation following estrogen stimulation, whereas wild-type epithelium recombined with ERα-negative uterine stroma did not proliferate (Cooke et al., 1997; Buchanan et al., 1998, 1999). These reports suggest that epithelial cell proliferation could be mediated indirectly by ERα in the stroma. In cell culture conditions, estrogen did not stimulate vaginal or uterine epithelial cell proliferation (Iguchi et al., 1983, 1985), however, estrogen stimulated DNA synthesis in human endometrial epithelial cell co-cultured with stroma cells in a transfilter system (Pierro et al., 2001). Estrogen stimulated vaginal and uterine stromal cell proliferation in culture (Inada et al., 2006). Thus, ERα activity in stromal cells is essential for estrogen-mediated epithelial cell proliferation in mouse reproductive tracts. Perinatal treatment with estrogens (e.g., E2, DES, EE2), aromatizable and non-aromatizable androgens, or BPA induce ovary-independent persistent proliferation of vaginal epithelium with superficial keratinization (Takasugi, 1976; Iguchi, 1992; Suzuki et al., 2002; Inada et al., 2006). No such changes in the vagina were induced in the neonatally DES-exposed αERKO mice (Couse and Korach, 2004), indicating the essential role of ERα in the induction of ovary-independent vaginal changes induced by estrogens. Here we showed that persistent vaginal epithelial cell proliferation with the superficial keratinization was induced by neonatal treatment with 0.25–2.5 μg DES or 25 μg PPT, but not DPN. Neonatal treatment with 0.025–2.5 μg DES or 2.5–25 μg PPT induced disorganization of circular muscle in the uterus; however, DPN did not induce this change. These results also indicate that ERα action is essential for induction of uterine muscular disorganization and ovary-independent persistent vaginal epithelial cell proliferation caused by estrogens during the critical developmental stage.

In the persistently proliferating vaginal epithelial cells in mice exposed neonatally to DES, phosphorylation of ERα and erbB2 receptor, and persistent expression of genes related to epidermal growth factor, such as amphiregulin (Areg), epiregulin (Ereg), heparin-binding EGF (HBegf), interleukin-1 (IL-1) receptor type II (Il1r2), IL-1 family member S (delta) (Il1f5), tumor necrosis factor-α and insulin-like growth factor-I have been reported (Miyagawa et al., 2004a,b). The present results show that the persistent expression of Areg, Ereg, Hbegf, Il1r2 and Il1f5 in vagina of mice treated neonatally with DES and PPT, but not DPN, results from ERα action, which is also essential for the induction of persistent molecular changes in the vagina.

Perinatal treatment with estrogens such as E2, DES, EE2 and genistein induces polyovular follicles (PFs) in the ovary (Iguchi, 1983; Iguchi and Takasugi, 1986; Iguchi et al., 1986, 1990; Jefferson et al., 2002; Kirigaya et al., 2006; Kipp et al., 2007). Neonatal treatment with a large dose of BPA also induced PFs in mice (Suzuki et al., 2002). The critical period for induction of PFs is within 3 days after birth in mice (Iguchi et al., 2002). ERα is localized in interstitial and thecal cells, whereas ERβ is localized in granulosa cells (Jefferson et al., 2002). ERβ is the predominant form in the ovary (Jefferson et al., 2002; Couse and Korach, 2004). ERβ is critical in granulosa cell differentiation and the ovulatory response to gonadotropins (Couse et al., 2005). Neonatal exposure of genistein induced PFs in wild-type and αERKO female mice, but not in βERKO females, and the induction of PFs in the ovary is dependent on the presence of
functional ERβ within the ovary (Jefferson et al., 2002). Our results show that neonatal treatment with PPT or DPN equally induce PFs in ovaries; therefore, both ERα and ERβ are involved in the induction of PFs. However, expression of ERα mRNA was higher than that of ERβ mRNA in the ovary of newborn mice. E2, progesterone and genistein disrupt nest breakdown and primordial follicle formation, which may result in loss of phosphorylation of Smad 2 protein, a marker of activin-dependent signaling, in the estrogen-treated ovaries. Therefore, both ER subtypes may be involved in these molecular and histological changes in the newborn mouse ovary.

In conclusion, neonatal treatment with DES or an ERα-selective ligand, but not an ERβ-selective ligand, induced anovulatory sterility, disorganization of uterine circular muscle and persistent proliferation of vaginal changes. We conclude that ERα action is essential for induction of abnormalities in the hypothalamic–pituitary axis and in the uterine and vaginal changes during critical developmental period in mice. In contrast, polyovular follicles were induced by neonatal treatment with both ERα and ERβ selective agonists; therefore, both ER subtypes are involved in induction of ovarian abnormalities induced by neonatal estrogenic chemicals.

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