Changes in ontogenetic expression of estrogen receptor alpha and not of estrogen receptor beta in the female rat reproductive tract

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Abstract

To evaluate ontogenetic expression and localization of estrogen receptor (ER) α and β in fetal female rat reproductive tract, competitive RT-PCR and immunohistochemistry were performed. Expression levels for Müllerian ERα, ERβ¹ and ERβ² mRNAs were determined by competitive RT-PCR. ERα expression on gestational day (GD) 15·5 increased 4·4-fold by GD 21·5, whereas both ERβ¹ and ERβ² gene expression were maintained at lower constant levels compared with ERα during development. ER immunolocalization was evaluated within three regions along the Müllerian duct axis; these were proximal, middle and caudal, which differentiate into oviduct, uterus and upper vagina respectively. Nuclear ERα was localized predominantly in proximal Müllerian epithelium, and middle and caudal Müllerian mesenchyme on GDs 15·5–21·5. Staining intensity for ERα increased with development in all regions. However, ERβ immunoreactivity was not detected in any region during prenatal life after separate staining with three different polyclonal anti-rat ERβ antibodies. These findings provide fundamental information critical for clarifying the species-specific physiological roles of ER subtypes during fetal development and for investigating the tissue-specific mechanisms underlying the prenatal response to estrogen and estrogen receptor agonists.

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Introduction

Estrogens are known regulators of cellular growth and differentiation in various target tissues. Estrogen actions are mediated through an estrogen receptor (ER) member of the nuclear receptor superfamily (Mangelsdorf et al. 1995). The ER–ligand complex binds target DNA at estrogen response elements (ERE) to transcribe various downstream genes such as oncogenes and genes encoding growth factors. In rats, two subtypes of ERs, classical ERα (Koike et al. 1987) and novel ERβ (Kuiper et al. 1996) have been identified. ERβ has also been cloned in mice (Tremblay et al. 1997) and humans (Mosselman et al. 1996), showing high homology between species. Estradiol (E₂) binds ERα with a higher affinity and increases ERα transcriptional activity at the ERE compared with ERβ in a reporter assay (Pettersson et al. 2000). Despite these differences in transcriptional activity, ERα and ERβ can have entirely opposite transcriptional effects at an AP-1 site, depending on
ligand (Paech et al. 1997). Moreover, the role of ERβ as a negative regulator of ERα has been suggested in the mouse uterus (Weihua et al. 2000).

Five isoforms of ERβ mRNA, ERβ1 (ERβ), ERβ2, ERβ1-83, ERβ2-83 and ERβ1-84, have been reported in various rat tissues as products of alternative splicing (Maruyama et al. 1998, Petersen et al. 1998, Price et al. 2000). Only ERβ1 and ERβ2, which has an additional 18 amino acids in the ligand binding domain of ERβ1, have the ability to bind both ligand and ERE (Maruyama et al. 1998, Petersen et al. 1998, Price et al. 2000). However, ERβ2 reportedly has apparently lower binding affinity for E2 compared with ERβ1 (Petersen et al. 1998). Therefore, ERβ2 can act as a dominant negative regulatory partner during heterodimerization with ERα or ERβ1 (Maruyama et al. 1998, Petersen et al. 1998), and this role of ERβ2 has been shown during rat mammary gland development (Saji et al. 2001). Since ERβ2 can act as a negative regulator of ERα and ERβ1, quantitative analysis of both estrogen receptor subtypes and isoforms may be key to understanding the role of ERs during development.

Insights from mice lacking genes for either or both ERs demonstrated the biological significance of each subtype in male and female reproduction (Lubahn et al. 1993, Krege et al. 1998, Couse et al. 1999, Muramatsu & Inoue 2000). The tissue distribution of ERα and ERβ in various organs has been reported in adult and fetal mice, rats and humans (Brandenberger et al. 1997, Couse et al. 1997, Saunders et al. 1997, Lemmen et al. 1999, Nishihara et al. 2000). Although expression of ERα and ERβ has also been reported in adult female reproductive organs (Shughrue et al. 1998, Hiroi et al. 1999, Sar & Welsch 1999, Wang et al. 1999, Mowa & Iwanaga 2000a, Pelletier et al. 2000), the onset, extent and patterns of expression during rat gestational and postnatal development are not well known.

Prenatal exposure to diethylstilbestrol (DES), a non-steroidal synthetic estrogen, causes a variety of abnormalities in the female reproductive organs such as oviduct uncoiling, uterine hyperplasia in addition to neoplasia, vaginal hyperplasia and cornification in mice and rats (Boylan 1978, McLachlan et al. 1980, Scully & Welch 1981, Newbold et al. 1983, Rothschild et al. 1987/88, Ozawa et al. 1991). Many estrogen agonists/antagonists including DES and environmental estrogens bind ERα and ERβ with different affinities (Kuiper et al. 1997, Petersen et al. 1998), suggesting ERα and ERβ play key roles in the developmental effects of these chemicals in target organs. However, strain differences in ERα ontogeny demonstrated in uterine epithelium of neonatal mice (Biggsby et al. 1990) indicate the need to define ER subtype expression in the reproductive tracts of other species, such as rat. In the present study, we performed quantitative temporal analyses of ERα, ERβ1 and ERβ2 gene expression levels during gestation by competitive RT-PCR. Furthermore, we determined the extent of ERα and ERβ protein localization along three defined regions within the fetal female rat reproductive tract using immunohistochemistry. Our findings clearly indicate tissue- and cell-specific differences in ERα and ERβ gene expression and protein localization during gestational development of the rat reproductive tract.

Materials and methods

Animals

Male and female Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Animals were housed individually in stainless-steel cages with controlled temperature (23 ± 2 °C) and relative humidity (55 ± 10%), and a 13-h light (0800–2100 h)/11-h darkness cycle. Pellet food (CRF-1, Oriental Yeast Co. Ltd, Tokyo, Japan) and municipal tap water were available ad libitum. Females were cohabited overnight with males. The day on which sperm was found in the vaginal smear was designated as gestational day (GD) 0. All animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

Primers

The following primers were used for competitive RT-PCR: primer 1, 5′-AAGAGAAGGACCACAT CCACC-3′ (forward primer for ERα); primer 2, 5′-GGAATGTGCTGAAGTGAGC-3′ (reverse
primer for ERα); primer 3, 5'-TTCTGGACAGGGATGAGGG-3' (forward primer for ERβ1); primer 4, 5'-GTCCTCAGAAAGACCTCACCTGAGG-3' (forward primer for ERβ2); primer 5, 5'-GGGACCTGTCACATGATTG-3' (forward primer for ERβ1 and ERβ2) and primer 6, 5'-GGGACACATTTCGACTT-3' (reverse primer for ERβ1 and ERβ2). Primers 1 and 2 (ERα), 3 and 6 (ERβ1), and 4 and 6 (ERβ2) were used for amplification and revealed 326 bp, 376 bp and 420 bp products respectively. For a comparison study of ERβ1 and ERβ2, primers 5 and 6 were used and revealed both 546 bp and 600 bp amplified products respectively.

**Total RNA preparation and competitive RT-PCR**

The fetal female reproductive tracts collected from three dams were placed in ice-chilled diethylpyrocarbonate (DEPC)-treated PBS, and gonads were dissected away under a dissecting microscope. Reproductive tracts were pooled from approximately 20 female fetuses on each gestational day and total RNA was isolated using TriZol (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA concentration was assessed by A260, and RNA was stored at −80 °C until needed.

Template total RNA (1 µg) was reverse-transcribed using SuperScript II RNase H− reverse transcriptase (Gibco-BRL) with oligo(dT)12–18 primer for 55 min at 70 °C and then chilled on ice. The generated cDNA was amplified with specific primers, various amounts of competitors constructed using the Competitive DNA Construction kit (Takara Shuzo Co. Ltd, Tokyo, Japan) and Taq DNA polymerase. The competitor has an intermediate sequence derived from DNA between the same two primer sequences used for the specific target gene. PCR cycle parameters were 95 °C for 60 s, 60 °C for 60 s and 72 °C for 120 s repeated for 25 and 35 cycles for ERα, and ERβ1 and ERβ2 respectively. The RT-PCR product was electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. Gel bands of amplified target and competitor were quantified by SCION image version 1.55 (Scion Co., Frederick, MD, USA) software and the ratio of target to competitor was calculated. Authenticity of all products was confirmed by sequencing with specific forward and reverse primers.

**Antibodies**

The monoclonal anti-human ERα antibody (NCL-ER-6F11) was obtained from Novocastra Laboratories Ltd, Newcastle upon Tyne, UK. A rabbit polyclonal anti-rat ERβ antiserum against a synthesized peptide (CSSTEDSKNKESQNLQSQ) corresponding to the C-terminal amino acid residues 467–485 of rat ERβ protein (Kuiper et al. 1996) was generated as described previously (Hiroi et al. 1999). The antiserum was purified by epoxy-activated Sepharose 6B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) coupled with the synthesized peptide. Two additional rabbit polyclonal antibodies to rat ERβ were evaluated. PA1-310 (Affinity Bioreagents, Inc., Golden, CO, USA) and 06-629 (Upstate Biotechnology, Lake Placid, NY, USA) are against the amino acid residues 467–485 and 54–71 of rat ERβ respectively. All three anti-ERβ antibodies are made against a region common between ERβ1 and ERβ2 and may bind both isoforms.

**Tissue preparation**

Pregnant and non-pregnant rats were killed by exsanguination from the abdominal aorta under ether anesthesia. Fetuses were collected on GDs 15.5, 17.5, 19.5 and 21.5. Ovaries and uteri from non-pregnant animals and fetuses were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C, dehydrated, paraffin embedded and sectioned to 6 µm. Changes in ER protein expression were studied within three designated regions along the axis of the Müllerian duct as per Visser et al. (1998). The proximal region exists at the level of the fetal ovary and differentiates into oviduct. The uterus arises from the middle region where the Müllerian and Wolffian ducts cross. The caudal region is near the urogenital sinus and differentiates into the upper vagina.

**Immunohistochemistry**

Ovarian, uterine and fetal reproductive tract tissue sections were deparaffinized, rehydrated and autoclaved at 121 °C for 15 min in 10 mM citrate buffer at pH 6.0. Sections were then rinsed three times in distilled H2O and incubated with 0.3% H2O2 in methanol for 30 min at room temperature (RT). After rinsing in PBS, sections were treated with 10% normal goat serum blocking solution.
(Zymed Laboratories, Inc., San Francisco, CA, USA) for 30 min at RT for ERα staining, or normal sheep serum (Dako Corporation, Carpinteria, CA, USA) overnight at 4 °C for ERβ staining. Next, sections were incubated overnight at 4 °C with anti-ERα antibody (diluted 1:50) or normal mouse IgG1 (DAKO A/S, Glostrup, Denmark) in 10% normal goat serum blocking solution for ERα staining, or for 48 h at 4 °C with anti-ERβ antibody (diluted 1:100 for our antibody and PA1-310, 10 µg/ml for 06–629) or normal rabbit immunoglobulin fraction (DAKO A/S) in normal sheep serum for ERβ staining. Anti-ERβ antibody binding specificity was evaluated in ovary and uterus by incubation with anti-ERβ antibody pre-absorbed with the synthesized immunizing peptide overnight at 4 °C. All sections were rinsed in PBS and treated with Simple Stain Rat PO (Nichirei, Tokyo, Japan), which is a horseradish peroxidase labeled polymer, for 1 h at RT. After a final PBS wash, sections were treated with 0.01% 3,3’-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris–HCl at pH 7.6 including 0.068% imidazole (Sigma Chemical Co., St Louis, MO, USA) and 0.02% H2O2 for 10 min at RT.

**Statistics**

Statistical analysis was carried out by Duncan’s multiple comparison test for ontogenetic expression of ERs. Differences in GD 19·5 Mullerian duct ERβ isoform expressions were evaluated by ANOVA and pairwise comparisons between PCR cycles were made using the Student’s t-test or Welch’s t-test in cases of equal variance or unequal variance, respectively. Data are reported as means ± S.D. and considered significantly different at P<0.05.

**Results**

**Competitive RT-PCR for ERα, ERβ1 and ERβ2**

Amplified products of expected size were detected by RT-PCR on GDs 15·5–21·5, but they were abolished by omitting the reverse transcriptase, and the authenticity of product sequence was verified by sequence analysis. To quantify expression levels of target genes, DNA competitors were amplified using the same primers as those for target genes. Various amounts of competitors for ERα were co-amplified with first strand cDNA of fetal female rat reproductive tracts from each gestational day (Fig. 1A) and the log ratios of target versus competitor were plotted against the log of competitor DNA copy numbers. The plotted curves are shown by linear regression (Fig. 1B).

Expression levels of ERβ1 and ERβ2 mRNAs were determined by the same procedure as ERα (data not shown).

Significant increases in ERα mRNA expression with fetal development were exhibited in the Mullerian duct. The ERα mRNA level on GD 21·5 was 4±4-fold higher than that on GD 15·5, but ERβ1 and ERβ2 mRNAs were lower than ERα mRNA and remained at similar constant levels from GD 15·5 through GD 21·5 (Fig. 2). Additionally, using PCR kinetics, comparison of ERβ1 and ERβ2 expression levels indicated ERβ2 mRNA expression is significantly greater than that of ERβ1 in the GD 19·5 Mullerian duct (P<0·01; Fig. 3).

**Immunohistochemistry for ERα**

Binding specificity of the anti-ERα antibody (6F11) to rat ERα has been evaluated previously (Fisher et al. 1997). In the present study, intense nuclear staining for ERα was observed in theca cells (Th), interstitial cells (In) and germinal epithelium (data not shown) in adult rat ovary (Fig. 4A). Also, in uterus, both luminal and glandular epithelia, and in stromal and myometrial tissues intense nuclear ERα staining occurred (data not shown). ERα staining in ovary (Fig. 4B) and uterus (data not shown) was abolished by incubation with normal mouse IgG1.

ERα immunostaining also appeared and was localized to nuclei within the Mullerian duct. In the proximal region on GD 15·5, mesenchymal ERα staining could be detected but epithelial staining appeared slight (Fig. 5A and Table 1). Epithelial and mesenchymal ERα levels within the proximal region increased gradually from GD 15·5 until GD 19·5 when levels became constant, with marked epithelial and only moderate mesenchymal staining through GD 21·5 (Fig. 5B–D and Table 1). In contrast, middle and caudal ERα levels continually increased within the mesenchyme throughout gestational development and reached marked intensity levels by GD 21·5 (Fig. 5E–L and Table 1).

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Figure 1 Competitive RT-PCR for estrogen receptor (ER)α. Reverse-transcribed total RNA isolated from the reproductive tract on gestational day (GD) 15.5, GD 17.5, GD 19.5 and GD 21.5 were co-amplified with serial-diluted competitor. The RT-PCR products were electrophoresed and visualized with ethidium bromide and UV transillumination (A). Upper and lower bands represent 326 bp ERα fragments and 441 bp competitor fragments respectively. The plotted curves are shown by linear regression (B). On the standard curve, the point where the log ratio equalled 0 (target/competitor=1) was considered as the concentration of reverse-transcribed mRNA.
However, epithelial ERα staining was absent or slight from the middle and caudal regions throughout gestation until GD 21·5 (Table 1). In addition, ERα immunostaining was absent in Wolffian epithelium and mesenchyme in all regions (Fig. 5A,B,E,F,I and J, and Table 1).

**Immunohistochemistry for ERβ**

Three different antibodies to ERβ were evaluated and each of these gave positive nuclear immunostaining in adult rat tissues. Intense staining for ERβ was seen in granulosa cells of the adult rat ovary (Fig. 4C–E). In uterus, ERβ immunoreactivity was moderate in glandular epithelium and myometrium, but slight in luminal epithelium and...
stroma (data not shown). ERβ staining in the ovary was abolished when tissues were incubated with normal rabbit immunoglobulin fraction in place of the primary antibody (data not shown) or anti-ERβ antibodies pre-neutralized with the synthesized ERβ peptides (Fig. 4F), confirming binding specificity. These observations on ERβ expression in adult rat ovary and uterus are consistent with those of previous reports (Hiroi et al. 1999, Wang et al. 1999, Nishihara et al. 2000, Pelletier et al. 2000). However, after immunostaining with each of three anti-ERβ antibodies, all three regions within the Mullerian or Wolffian ducts appeared negative for ERβ throughout gestation (data not shown).

Figure 4 Immunohistochemical localization of ERα and ERβ in adult rat ovary. ERα nuclear staining (A) is seen in theca cells (Th), interstitial cells (In) and germinal epithelium (data not shown). For ERβ, positive nuclear staining is seen in granulosa cells (Gr) of ovary with our antibody to rat ERβ (C) and with anti-rat ERβ antibodies from Affinity Bioreagents (D) and Upstate Biotechnology (E). ERα and ERβ immunoreactivities were abolished by incubation with control IgG1 (B), or pre-absorbed anti-ERβ antibodies (ours, F; others, data not shown) respectively. Bar: 100 µm.
Discussion

Ontogenetic localization and quantitative analysis of ER\(\alpha\) and ER\(\beta\) expression were investigated in the fetal female rat reproductive tract. Using competitive RT-PCR and immunohistochemistry, fetal female reproductive tract ER\(\alpha\) is expressed exclusively in the Müllerian duct and is detectable by at least GD 15.5. Overall, ER\(\alpha\) expression levels continue to increase with gestational development. Expression of both ER\(\beta1\) and ER\(\beta2\) was apparently lower than that of ER\(\alpha\) throughout fetal development, indicating ER\(\alpha\) is a dominant receptor subtype in the Müllerian duct. Additionally, important region-specific changes in ER expression are defined.

Immunohistochemistry for ER\(\alpha\) and ER\(\beta\) showed differential expression of the two ER subtypes in ovary and uterus of adult rats. These observations are in agreement with previous reports (Shughrie \textit{et al.} 1998, Hiroi \textit{et al.} 1999, Sar \& Welsch 1999, Wang \textit{et al.} 1999, Mowa \& Iwanaga 2000\textit{a}, Pelletier \textit{et al.} 2000), and confirmed antibody specificity. However, despite the findings in adult rodents, in this study we demonstrated region-specific ER\(\alpha\) localization in the fetal rat Müllerian duct. Furthermore, region-specific ER\(\alpha\) expression firmly indicates that functional differentiation within the rat Müllerian duct occurs before morphological differentiation after birth, and suggests regional targets for chemicals that may act via or influence estrogen receptor-mediated mechanisms during late gestational and potentially early neonatal Müllerian duct differentiation.

Region-specific expression of ER\(\alpha\) was previously reported in the female reproductive tract in neonatal mice. On the day of birth, ER\(\alpha\) was expressed in epithelial cells of mouse oviduct, cervix and vagina, but not in uterus. In contrast, stromal cells expressed ER\(\alpha\) in these organs including uterus (Yamashita \textit{et al.} 1989, Bigsby \textit{et al.} 1990). However, strain differences in the ontogenetic localization of ER\(\alpha\) were reported in uterine epithelial cells in neonatal BALB/c and CD-1 mice (Bigsby \textit{et al.} 1990), suggesting the importance of the study on ER expression in the reproductive tracts of each strain and species.
In the fetal case, ERα is expressed in the Müllerian duct of mice (Greco et al. 1991, Lemmen et al. 1999, Jefferson et al. 2000, Nielsen et al. 2000) and rats (Mowa & Iwanaga 2000b). Greco and co-workers (1991) reported that ERα immunoreactivity was observed in epithelial cells of female mouse reproductive tract on GD 15, but was occasionally observed on GD17 and the day of birth. Likewise, expression of ERα was detected only in female Müllerian mesenchyme (Lemmen et al. 1999). However, since the results of Nielsen et al. (2000), Mowa and Iwanaga (2000b), and the present study demonstrate that ERα localization varies with reproductive tract region, it seems appropriate to examine the reproductive tract at a variety of specific regions.

Prenatal mouse and rat reproductive tract is negative for ERβ mRNA (Lemmen et al. 1999, Jefferson et al. 2000, Mowa & Iwanaga 2000b), but ERβ mRNA was detected by RT-PCR in uterus of the midgestational human fetus (Brandenberger et al. 1997, Takeyama et al. 2001). Takeyama et al. (2001) demonstrated that ERβ is the dominant subtype during fetal development of humans. These observations suggested species differences in ERβ expression in the fetal reproductive tract. In the present study, rat Müllerian duct ERβ1 and ERβ2 mRNAs were detected and quantitated by competitive RT-PCR and found to be expressed at constant levels from GD 15.5 to GD 21.5. However, expression levels of ERβ1 and ERβ2 mRNA were much lower than that of ERα. Therefore, since ERβ protein may also be at a low level, it may occur at levels below the limit of immunohistochemical detection. Moreover, the immunostaining results that we obtained using two additional commercially available polyclonal anti-rat ERβ antibodies (PA1–310 and 06–629) were virtually indistinguishable from those presented here. These results indicate that ERα is probably a dominant ER subtype during rat Müllerian duct development.

Interestingly, ERβ2 expression was higher than that for ERβ1 in the Müllerian duct. Although tissue-specific ratios of ERβ1 and ERβ2 expression have been described in various rat tissues – approximately 1:1 in prostate, ovary, muscle and pituitary, and 2- to 6-fold expression of ERβ1 compared with that of ERβ2 in tissues of the nervous system – there have been no reports of a tissue in which ERβ2 is higher than ERβ1 (Petersen et al. 1998). Recently, the important role of ERβ2 as a negative regulator of ERα has been shown in the rat mammary gland in which ERβ2 levels are comparable or higher than those for ERβ1 (Saji et al. 2001). Although both ERβ1 and

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++, marked staining; +, moderate staining; ±, slight staining; —, not detectable; NF, not formed.
ERβ2 levels are obviously higher than ERα in mammmary gland, both isoforms were extremely low compared with ERα in rat Müllerian duct, implying insufficient amounts of ERβ2 as a negative regulator of ERα in rat Müllerian duct. The physiological significance of ERβ2 in rat Müllerian duct is unknown, but different ratios of ERα/ERβ1/ERβ2 between various tissues may suggest tissue-specific regulation via ER subtypes and isoforms.

Teratogenic effects of a prenatally administered estrogen agonist such as DES on the female reproductive tract have been demonstrated in rodents (Boylan 1978, McLachlan et al. 1980, Scully & Welch 1981, Newbold et al. 1983, Rothschild et al. 1987/88, Ozawa et al. 1991). DES binds ERα and ERβ with higher affinity than E2 (Kuiper et al. 1997), and prenatal DES exposure is thought to act via ERs in the fetal female reproductive tract (Greco et al. 1993). Indeed, autoradiography revealed extensive binding by [3H]DES in mouse reproductive tract on GD 17 following transplacental exposure (Holderegger & Keefer 1986). In our previous report, we suggested that DES may act mainly via ERβ, since DES down regulated ERβ1 and ERβ2 mRNAs, but not ERα mRNA (Okada et al. 2001). Based on the present findings, however, transplacental exposure to estrogen agonists including DES may act in the Müllerian duct via dominantly expressed ERα. Also, tissue-specific effects of DES on cell proliferation in the Müllerian duct (Okada et al. 2001) may be influenced by tissue-specific expression of ERα.

In conclusion, ontogenetic expression of ERα, ERβ1 and ERβ2 in the fetal rat reproductive tracts is defined. The patterns of ERα localization suggest potential tissue-specific mechanisms by which estrogenic chemicals may influence Müllerian duct cell growth and differentiation. These observations may be helpful in clarifying the mechanism of estrogen action including environmental estrogens. The relative physiological significance of ER subtypes and isoforms in the prenatal female reproductive tract remains to be further evaluated.

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