Ontogenetic Changes in the Expression of Estrogen Receptor α and β in Rat Pituitary Gland Detected by Immunohistochemistry

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ABSTRACT

The physiological effects of estrogen on the pituitary, including cellular proliferation and regulation of hormone synthesis, are mediated by the nuclear estrogen receptor (ER). The purpose of this study was to determine ontogenetic expression of two types of ERs (ERα and ERβ) in the pituitary using specific antibodies, monoclonal antibody (1D5) for ERα and polyclonal antibody generated against ERβ. First, we confirmed the detection of 66- and 55-kDa bands for ERα and ERβ, respectively, in the rat pituitary extract by Western blotting. Then immunostaining with these antibodies was performed using fetal and adult Wistar rat tissues, combined with PRL or LHβ immunohistochemistry. Intense ERβ signal was detected throughout the pituitary from day 12 of gestation. However, staining for ERα only became detectable from day 17 of gestation. In contrast with the fetal period, nuclei stained for ERα were widely distributed in the anterior lobe in the adult rat, whereas ERβ-positive cells were restricted in the anterior lobe. LHβ, but not PRL, was colocalized in ERβ-positive cells. Our results indicated that the major population of ER subtypes in the pituitary gland has changed around the day of birth and that the expression of ERβ may be involved in the differentiation of pituitary cell function to synthesize a specific hormone. (Endocrinology 141: 615–620, 2000)

ESTROGEN PLAYS various roles in the pituitary, including cellular proliferation and regulation of hormone synthesis, and its action is mediated through the nuclear estrogen receptor (ER). Several pituitary cells, such as lactotrophs (1, 2) and gonadotrophs (3, 4), are directly regulated by estrogen. The expression of ERα (original type) in the pituitary has been investigated in detail by using immunohistochemistry (5–7), in situ hybridization (8, 9), and RT-PCR (10). ERα messenger RNA (mRNA) has been demonstrated in most cells of the intermediate and anterior lobes of the pituitary by in situ hybridization (8). Furthermore, ERα immunoreactivity has been identified in many anterior pituitary cell types, including gonadotrophs and lactotrophs (6, 7).

After cloning a novel ER, ERβ (11), Kuiper et al. (12) reported a low expression of ERβ mRNA in the adult rat pituitary by RT-PCR. Although the distribution of ERβ mRNA in the adult rat pituitary has been recently examined by in situ hybridization (13–15), it remains to be determined whether the expression of ERβ mRNA is restricted to the anterior lobes or to both anterior and intermediate lobes. In addition, the localization of ERβ protein in the pituitary remains to be determined.

Wilson et al. (13) recently reported that the expression level of ERβ mRNA in the prepubertal rat pituitary is higher than that in the adult rat. Although the fetus in placental mammals is exposed to relatively high levels of maternal and placental estrogens (16), it is not clear whether ERβ is expressed in the rat fetal pituitary and whether the expression pattern changes during fetal life.

The functional roles of ERβ have been recently reported by analyzing ERβ target cells in the adult rat pituitary. For example, colocalization of ERβ in gonadotrophs and lactotrophs has been demonstrated using dual immunocytochemistry/in situ hybridization (13, 14). However, it is still controversial that gonadotrophs are the direct target cells for ERβ. Although ERβ can form homodimers and heterodimers with ERα in vitro (17), Mitchner et al. (14) reported only a small percentage of ERβ colocalizes with ERα in the pituitary.

In the present study we examined the cellular distribution of ERα and ERβ using immunohistochemistry with autoclave-antigen retrieval in sections of fetal and adult rat pituitary glands. The antibody for ERβ was raised by immunizing rabbits with a synthetic peptide of a part of rat ERβ, and the specificity of this antibody was confirmed by Western blotting. Our results showed a dramatic change in the major ER population from ERβ to ERα in the pituitary around the day of birth. Finally, in combination with immunohistochemistry for PRL and LHβ, we demonstrated a significant role for ERβ in the gonadotroph, but not in the lactotroph.

Materials and Methods

Animals

Wistar rats were maintained at the Nagasaki University animal facility. All experiments were conducted according to the principles and
procedures outlined in the Guideline for the Care and Use of Laboratory Animals of Nagasaki University School of Medicine. For tissue sampling, fetuses on embryonic days (E) 12, 14, and 17 were dissected free from the dam under light ether anesthesia. Rats on postnatal day (P) 1 and at 8 weeks were decapitated, and the pituitary, prostate, and ovaries were rapidly excised. Adult female rats were obtained with random estrous cycling.

Preparation of antibody

ERα was detected with 1D5 antibody (DAKO Corp., Glostrup, Denmark). For the detection of ERβ, a peptide (CSSTEDSKNKESQNLQSQ) corresponding to the C-terminal amino residues of rat ERβ (11) was conjugated to keyhole limpet hemocyanin. Then, the rabbit polyclonal antirat ERβ antiserum was generated by immunizing rabbits followed by purification as described previously (18). Specific polyclonal antibody to PRL was obtained from Biogenesis (Bournemouth, UK), and antibodies to GH, TSH, and LHβ were gifts from Dr. Wakabayashi (Gunma University, Maebashi, Japan).

Western blotting

Pituitary and ovarian tissues from 8-week-old rats were excised and immediately frozen, followed by preparation for sampling, as previously described (14). Thirty micrograms of each protein were separated on SDS-PAGE (7.5% polyacrylamide gels) and then transferred to nitrocellulose membranes by electroblotting. After blocking with 10% nonfat milk in Tris-buffered saline (TBS) buffer overnight at 4°C, blots were incubated for 1 h with the monoclonal antibody against ERα at a 1:200 dilution in 10% nonfat milk and preincubated with 500 μl/ml goat IgG and 5% BSA in PBS for 60 min at room temperature to reduce nonspecific binding of antibodies. Sections were then reacted with the primary antibody diluted at 1:100 for 1 h at room temperature. After washing with 0.075% Brij 35 in PBS, sections were incubated with HRP-goat antirabbit or antiperoxidase-conjugated secondary antibody (MBL, Nagoya, Japan) at a 1:100 dilution in TBS/0.05% Triton X buffer. The membranes were subsequently washed three times for 10 min each time with TBS/0.05% Triton X buffer. Each membrane was treated with either goat antirabbit or antimouse IgG-peroxidase-conjugated secondary antibody (MBL, Nagoya, Japan) at a 1:100 dilution for 1 h in the monoclonal antibody against ERα at a 1:500 dilution or the polyclonal antibody against ERβ at a 1:100 dilution in TBS/0.05% Triton X buffer. The membranes were subsequently washed three times for 10 min each time with TBS/0.05% Triton X buffer.

Immunohistochemistry

Tissues were fixed with 4% paraformaldehyde in PBS for 12 h, processed for 24 h in a tissue processor, and embedded in paraffin. Each 5-μm thick tissue section was cut and mounted on silane-coated slides. For ERα and ERβ immunohistochemistry, sections were dewaxed, rehydrated, and autoclaved at 120°C for 10 min in 10 mM citrate buffer (pH 6.0) (20). After washing in PBS, endogenous peroxidase was blocked using 0.3% H2O2 in methanol (15 min). Slides were washed in PBS again and preincubated with 500 μg/ml goat IgG and 5% BSA in PBS for 60 min at room temperature to reduce nonspecific binding of antibodies. Sections were then reacted with the primary antibody diluted at 1:200 for ERα and at 1:100 for ERβ overnight at room temperature. After washing in 0.075% Brij 35 (Sigma, St. Louis, MO) in PBS, sections were incubated with horseradish peroxidase (HRP)-goat antimouse IgG or HRP-goat antirabbit IgG at a 1:100 dilution for 2 h at room temperature, respectively. After washing in 0.075% Brij 35 in PBS, the sites of HRP were visualized by DAB, H2O2, Co2+, and Ni2+ using methyl green as a counterstain.

Western blot analysis of ERα and ERβ. Thirty-microgram extracts from adult rat (8-week-old) pituitary and ovary tissues were subjected to SDS-PAGE. Western blotting was performed using a monoclonal antibody (1D5) for ERα and a polyclonal antibody generated against ERβ. The arrow indicates the 55-kDa band.

Preparation of antibody

For GH, TSH, LHβ, and PRL immunohistochemistry, sections were dewaxed and rehydrated, and endogenous peroxidase was blocked, using the same method described above for ERα and ERβ immunohistochemistry. After washing with PBS and blocking, sections were reacted with each primary antibody diluted at 1:1000 for 1 h at room temperature. After washing in 0.075% Brij 35 in PBS, sections were incubated with HRP-goat antirabbit IgG at a 1:100 dilution for 2 h at room temperature. After washing in 0.075% Brij 35 in PBS, sites of HRP were visualized by DAB and H2O2 using methyl green as a counterstain.

Negative controls were prepared by reacting a few sections with normal mouse IgG or normal rabbit IgG at the same dilution instead of the specific antibody.

Image analysis

Photographs of immunostained sections around sinusoid vessels in the anterior lobes of the pituitary were selected from separate sections from three adult male rats. After selecting the area of tissue section...
stained for ERβ, the corresponding area in mirror sections stained for ERα, PRL, and LHβ was photographed. One negative control section for each paired section was also photographed. The percentages of ERα, PRL, and LHβ with ERβ colocalized in the pituitary were calculated among 100 cells, which were not stained in the negative control, for each animal.

Statistical analysis

All data were expressed as the mean ± sd. Differences between groups were examined for statistical significance using Student’s t test. P < 0.05 denoted the presence of a statistically significant difference.

Results

Western blot analysis of ERα and ERβ

To confirm the specificity of the antibodies used for ERα and ERβ, Western blot analysis of extracts from the rat ovaries, which are known to express both ERα and ERβ, was performed. As shown in Fig. 1, 66- and 55-kDa bands were detected with anti-ERα antibody (1D5) and anti-ERβ antibody, respectively. When the extract of the adult rat pituitary was analyzed by Western blotting in the same way, we also
found 66- and 55-kDa bands for ERα and ERβ, respectively. When the first antibody was omitted, no bands were observed (data not shown).

**Immunohistochemistry**

To assess the specificity of these antibodies under our standard protocol of immunohistochemistry, we first detected the signals for ERα and ERβ in positive tissues, uterus and prostate, respectively. Staining for ERα was detected in the nuclei of glandular epithelium and stromal cells in the endometrium of the uterus (data not shown), whereas ERβ immunoreactivity was detected in nuclei of secretory epithelial cells of the prostate (Fig. 2A).

In the next step, we examined the pituitary glands of fetal rats for the expression of ERα and ERβ. ERα immunoreactivity was not detected in Rathke’s pouch from E12 to E14 (data not shown), but appeared in a small population of anterior lobe cells on E17 (Fig. 3, A and B). In the adult male rat (8-week-old), nuclear staining for ERα was detected in a large population of anterior lobe cells, but was not detected in the intermediate lobe or posterior lobe (Fig. 4). In contrast, the signal for ERβ was detected as intensely stained dots inside Rathke’s pouch on E12 (Fig. 3C). Furthermore, immunopositive cell regions changed during development from the internal side of Rathke’s pouch to the anterior and posterior lobes (Fig. 3, C and D–F). In other regions of the brain, the choroid plexus and olfactory epithelium were also positive for ERβ from E17 onward. On P1, staining of the choroid plexus did not change, but olfactory and nasal epithelia showed more abundant signals, and the taste buds also became positive for ERβ (data not shown).

In the adult male rat, ERβ-positive cells were distributed mainly in the periphery and sex zone (area adjacent to the intermediate lobe) of the anterior lobe (Fig. 4), with fewer such cells in the intermediate lobe. In the posterior lobe, ERβ-positive cells were also fewer than in the anterior lobe, and most staining shown in Fig. 4 represented nonspecific signals in nerve fibers. Interestingly, the signal for ERβ, unlike that for ERα, was distributed in the nucleus, perinuclear region, and cytoplasm in positive cells in the fetal pituitary (Fig. 2, B and C). However, in the adult pituitary, it was detected mostly in the nuclei (Fig. 2D). For both ERs, no differences in the expression pattern were detected between male and female rats.

Hormonal expression, including GH, PRL, TSH, and LHβ, in the pituitary was detected from P1 (data not shown). The largest population among pituitary cells was GH cells, and the smallest one was LHβ cells. However, these hormonal expressions could not be detected until E17, in which all sections were just adjacent to the sections examined for ERβ expression.

To determine the cell types that coexpressed ERβ in the pituitary of male adult rats, we performed immunohistochemistry for ERβ, ERα, LHβ, and PRL proteins in mirror sections (Fig. 5). Measurement of the percentage of cells that colocalized ERβ among LHβ- or PRL-positive cells in the anterior lobes showed that 67.2 ± 5.3% (mean ± SD; n = 3) of LHβ- and 11.2 ± 0.2% (n = 3) of PRL-positive cells coexpressed ERβ protein, and the difference was statistically significant (P < 0.001). In addition, the percentage of ERα-positive cells in the anterior lobes that colocalized ERβ protein were 20.6 ± 2.1% (n = 3).

In the negative control, reaction of sections with normal IgG at the same dilution instead of the specific antibody showed no staining above the background in any fetuses regardless of the developmental stage (Fig. 3, G and H–J) and in the adult period (data not shown).

**Discussion**

We described in the present study the distribution of ERα and ERβ in the fetal and adult rat pituitary immunohistochemically. The major findings of our study were the following. 1) The expression of ERβ protein in the fetal rat pituitary gland is higher than that of ERα. 2) The major population of ER subtypes has changed in the pituitary around the day of birth. 3) The cellular distribution of ERβ is not clear during fetal life. 4) In the adult pituitary, the expression of ERβ protein is largely confined to gonadotroph cells.

Estrogen binding is detected in the rat brain from E21 (21) and in the mouse pituitary from E17 (22). The rat glucocorticoid receptor mRNA, such as the nuclear hormone receptor

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**Fig. 4.** Adult (8-week-old) male rat pituitary immunostained for ERα and ERβ using specific antibodies. Insets show peripheral anterior lobe cells. Magnification for pituitary sections, ×12.5; insets, ×400.
ER, is present in Rathke’s pouch from E13 (23). In the mouse fetus, various transcription factors, such as Ptx1, Prop1, Pit1, and SF1, present in Rathke’s pouch from about E12 influence the development of pituitary gland (24). Therefore, in our study we analyzed the expression of ERs in the rat pituitary from E12 to adulthood by immunohistochemistry. First, we showed that the expression of ER\(\alpha\) in the fetal pituitary was lower than that during the adult period. Furthermore, we showed that the expression of ER\(\alpha\) was detected in the nuclei of the anterior lobe cells from E17, demonstrating that this event occurs in an earlier period of fetal life than that was previously reported (25). In contrast, a high expression of ER\(\beta\) was detected in Rathke’s pouch from E12, and the distribution of ER\(\beta\) spread from inside Rathke’s pouch to the anterior and posterior lobes during the fetal period. This finding is in agreement with that reported in the human midgestational fetus, where high amounts of ER\(\beta\) mRNA were described in the pituitary by RT-PCR (26). The distribution of ER\(\beta\) in the adult was mainly restricted in clusters in the anterior lobe. These findings suggest that the main ER subtypes in the rat pituitary change around the day of birth. The exact role of ER\(\beta\) in the pituitary during the fetal period and the functional significance of high expression remain unknown at present. Furthermore, the mechanisms leading to the change in the major ER population from ER\(\beta\) to ER\(\alpha\) are not clear.

In the adult, our study showed that the expression of ER\(\beta\) was restricted to the anterior lobes. Gonadotrophs are distributed in clusters around sinusoid vessels in the anterior lobe, including the sex zone (27). Our results showed that the distribution of ER\(\beta\) was similar to that of the gonadotroph in the adult pituitary. We also analyzed the adult pituitary cell types that colocalize ER\(\beta\) and LH\(\beta\) or PRL. Our results showed that 67% of LH\(\beta\)-positive cells were also positive for ER\(\beta\), whereas only 11% of PRL-positive cells were positive for ER\(\beta\). These findings suggest that the direct target cells for ER\(\beta\) in the pituitary are more gonadotrophs than lactotrophs. In agreement with this finding, Wilson et al. (13) reported that

**Fig. 5.** Colocalization of ER\(\alpha\), PRL, and LH\(\beta\) with ER\(\beta\) in adult (8-week-old) male rat pituitaries. Pituitary sections were immunostained for ER\(\alpha\), PRL, and LH\(\beta\) (left panels) and ER\(\beta\) (right panels) in mirror sections. Each cell marked by an arrowhead in the left panel corresponds to the cell indicated by the arrowhead in the right panel, respectively. Arrowheads indicate ER\(\beta\)-positive cells that did not express ER\(\alpha\) (upper panels), ER\(\beta\)-positive cells that expressed LH\(\beta\) (middle panels), and ER\(\beta\)-negative cells that expressed PRL (bottom panels). Magnification, \(\times\)400; insets, \(\times\)1000.
Although ERβ mRNA was not expressed in the lactotrophs, it was expressed in 85% of FSH-positive cells. Although ERβ/ERα heterodimers are preferentially formed over ERβ homodimers (17), only 21% of ERα-positive cells, which were widely spread in the anterior lobes of adult rats, coexpressed ERβ. These findings suggest that the majority of cells expressing ERβ may act specifically with ERβ homodimers in the pituitary.

In our studies, ERβ immunoreactivity in the fetal pituitary was detected in both the nucleus and cytoplasm, whereas ERα immunoreactivity was limited to the nuclei of anterior lobe cells. As we confirmed that ERβ was solely localized in the nuclei of rat ovary and uterus using the same antibody, our findings could be considered a specific feature of ERβ distribution in pituitary cells. Moreover, from around P1, we detected GH, PRL, TSH, and LHβ expression in the pituitary and the major population among these hormones in this period was LHβ-positive cells, in accordance with previous reports (28, 29). In contrast, ERβ expression has been detected from E12. Considering the close connection of LHβ-positive cells with ERβ expression in the adult, ERβ might play different roles in the pituitary during the fetal and adult periods.

In summary, the specific antibodies for ERs used in our study are useful for identifying both forms of ERs in the fetal and adult rat pituitaries. Our results of ER subtype change in the major population around the day of birth may play a role in the differentiation of pituitary cells, but functional studies are necessary for full understanding of ERβ function. In the adult rat, gonadotroph-specific expression of ERβ indicates that estrogen may regulate the function of gonads through ERβ.

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