Differential immunolocalization of estrogen receptor α and β in rat ovary and uterus

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ABSTRACT

In order to investigate the localization of estrogen receptor (ER) α and ERβ in the reproductive organs in the rat, polyclonal antibodies were raised to each specific amino acid sequence. The Western blot with anti-ERα antibody showed a 66 kDa band in rat ovary and uterus, while that with anti-ERβ antibody detected a 55 kDa band in rat ovary, uterus and prostate. The ligand-independent nuclear localization of the two receptors was verified by immunocytochemistry. By immunohistochemistry, the nuclei of glandular and luminal epithelial cells in the uterus were stained with anti-ERα antibody, whereas only the nuclei of glandular epithelium cells were stained with anti-ERβ antibody. In rat ovary, positive signals were shown with anti-ERβ antibody in the nuclei of granulosa cells. No specific immunostaining was observed with anti-ERα antibody. Although ERβ was immunostained at the proestrous, metestrous and diestrous stages, the immunoreactivity of ERβ was hardly detected at the estrous stage in rat ovary. Thus, we show differential expression of ERα and ERβ in rat uterus and ovary at the protein level, which may provide a clue for understanding the roles of the two receptors in reproductive organs.

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INTRODUCTION

The estrogen receptor (ER), a member of the steroid/thyroid hormone receptor superfamily (Mangelsdorf et al. 1995), is a ligand-dependent transcription factor. It specifically binds with estrogen, and regulates gene transcription via the estrogen responsive element (ERE). ER had been assumed to exist as a single species (Green et al. 1986, Greene et al. 1986, Koike et al. 1987), until a novel estrogen receptor (ERβ) was recently isolated in rats (Kuiper et al. 1996), humans (Mosselman et al. 1996, Ogawa et al. 1998), and mice (Tremblay et al. 1997). ERβ has a high degree of sequence homology with the classical estrogen receptor (ERα). ERβ mRNA was detected predominantly in rat ovary, prostate, lung, brain, bladder, uterus and bone (Kuiper et al. 1996, 1997, Shuhurue et al. 1996, Onoe et al. 1997), and in human breast cancers (Dotzlaw et al. 1997). Although ERβ has a slightly lower binding affinity for 17β-estradiol than ERα (Kuiper et al. 1996, 1997, Tremblay et al. 1997), its transactivating manner via ERE is similar to ERα (Pace et al. 1997, Pettersson et al. 1997). On the other hand, some transactivating functions of ERβ may be different from those of ERα, which depend on the ligand and responsive element (Peach et al. 1997, Tremblay et al. 1997, Watanabe et al. 1997).

To understand the differential functions of these molecules, determination of precise localization, especially at the protein level, remains to be determined. Therefore, we have generated antibodies specific to rat ERα and ERβ and investigated their localization in reproductive organs in rats by Western blot analysis and immunohistochemistry.

MATERIALS AND METHODS

Animals

Female and male Sprague-Dawley rats aged 8–12 weeks were used in this study. All animals were
maintained in accordance with the institutional guidelines for care and use of laboratory animals. For the Western blot analysis, the ovary, uterus, prostate and testis were removed, rapidly frozen and stored in liquid nitrogen until use. For immunohistochemistry, the ovary and uterus were removed and soaked in neutral formalin, and then embedded in paraffin. Twenty-micron-thick sections were prepared for immunohistochemistry. The stage of the estrous cycle was determined by vaginal cytology.

**Antibody preparation**

We synthesized peptides CSLQTYYIPPEAEGFPNTI and CSSTEDSKNKESSLQSQ, the specific C-terminal amino acid residues of predicted rat ERα (Koike et al. 1987) and ERβ (Kuiper et al. 1996) protein respectively. These synthesized peptides were coupled with keyhole limpet hemocyanine (KLH) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). The rabbit polyclonal anti-rat ERα antiserum and anti-rat ERβ antiserum were generated by subcutaneous injection of the KLH which was coupled with the synthesized peptide and emulsified in complete Freund’s adjuvant. The antiserum was purified by 2-fluoro-1-methylpyridinium toluen-4-sulfate (FMP)-activated cellulofine (Seikagaku-kogyo, Tokyo, Japan) and was used at a concentration of 1:1000 using the ECL system (Amersham, Tokyo, Japan).

The subcellular localization of rat ERα and ERβ protein was examined as described (Ikegami et al. 1994). Briefly, 293T cells, transfected with each expression vector, were reseeded and allowed to grow to subconfluence on chambered glass slides. The cells were fixed with acetone and methanol (1:1) and blocked by 2% normal goat serum in phosphate-buffered saline (PBS) at room temperature for one hour. The cells were incubated with the purified antiserum (1:100). Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (IgG) was used as the second antibody. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The stained cells were observed under immunofluorescence microscopy.

**RESULTS**

**Western blot analysis of rat ERα and ERβ**

Polyclonal antibodies specific for rat ERα and ERβ were generated using the synthesized peptide. The anti-ERα antibody reacted with three bands of 66 kDa, 55 kDa and 50 kDa in the nuclear extracts of 293T cells transfected with the rat ERα expression vector. The Western blot analysis detected a 66 kDa band which reacted with the anti-ERα antibody in the ovary and uterus, but not...
in the prostate and testis (Fig. 1A). Using the anti-ERα antibody absorbed with the original synthesized peptide, the immunoreactive band was not observed (Fig. 1B).

The anti-ERβ antibody reacted with doublet bands of 56 and 55 kDa in the nuclear extracts of 293T cells transfected with rat ERβ expression vector. Other immunoreactive bands were seen in the nuclear extracts of 293T cells transfected with pSSRa vector and 293T cells transfected with rat ERα expression vector. Smaller bands were detected in the nuclear extracts of 293T cells transfected with rat ERβ expression vector. These smaller bands were not detected in the nuclear extracts of rat tissues. An immunoreactive band with anti-rat ERβ antibody was detected at approximately 55 kDa in the ovary, uterus and prostate, but not in the testis. About 60 kDa immunoreactive bands were observed in the ovary, uterus and prostate (Fig. 1C). Using the anti-ERβ antibody absorbed with the original synthesized peptide, the immunoreactive band was not observed (Fig. 1D).

Nuclear localization and immunohistochemistry of ERα and ERβ

The ERα and ERβ immunoreactivities were detected in nuclei of 293T cells transfected with rat ERα and ERβ expression vectors respectively. The immunoreactive products for ERα and ERβ in 293T cells reside in nuclei regardless of the presence of E2 at $10^{-8}$ M (Fig. 2). Immunoreactivity of ERα was not detected in 293T cells transfected with rat ERβ expression vector and vice versa and no immunoreactive signals were detected in untransfected 293T cells (data not shown).

In rat uterus, immunoreactivity for ERα was observed in the nuclei of glandular epithelium and luminal endometrium (Fig. 3A, B). On the other hand, anti-ERβ antibody immunoreactive products were confined to the nuclei of glandular epithelium (Fig. 3E). No staining was detected when pre-immune serum was used (Fig. 3G, H). The immunostaining disappeared when the antibodies absorbed with the respective original synthesized peptides were used (Fig. 3C, F).

Immunohistochemistry of rat ovary stained with anti-ERβ antibody showed immunoreactivity in the nuclei of granulosa cells of follicles (Fig. 4C). No staining was detected when anti-rat ERα antibody (Fig. 4A) was used. The signals disappeared when the antibodies were absorbed with the synthesized peptides (Fig. 4B, D). The expression of ERβ protein through the estrous cycle in the rat ovary was examined by immunocytochemistry (Fig. 5). The immunoreactivity of ERβ was observed in the nuclei of granulosa cells of follicles at the proestrus (Fig. 5A), metestrus (Fig. 5C) and diestrus (Fig. 5D) stages. At the estrous stage, the signal of ERβ
was hardly detected by immunocytochemistry (Fig. 5B).

**DISCUSSION**

The specific antibodies for rat ERα and ERβ were raised and used for the detection of the differential expression of ERα and ERβ in rat reproductive organs. The ERβ bands were observed in the nuclear extracts of rat ovary, uterus and prostate, but not testis. A previous paper reported the expression of rat ERβ mRNA in the ovary, uterus, prostate and testis by reverse transcription (RT)-PCR (Kuiper et al. 1997). The amount of ERβ protein in the testis may be too small to detect under the conditions employed here.

Western blot analysis detected ERβ immunoreactive doublet bands of 56 and 55 kDa in 293T cells transfected with rat ERβ expression vector. Another band (NS in Fig. 1) was detected below these bands. This band was also positive in 293T cells transfected with a vector alone or transfected with rat ERα expression vector, suggesting that there were some products in the nuclei of 293T cells detectable with the anti-ERβ antibody used in this study.

![Figure 2. Nuclear localization of ERα and ERβ. 293T cells transfected with rat ERα expression vector were stained with anti-rat ERα antibody (A and C) and DAPI (B and D). 293T cells transfected with rat ERβ expression vector were stained with anti-rat ERβ antibody (E and G) and DAPI (F and H). 293T cells were grown without (E2(-): A, B, E and F) or with 17βestradiol (10⁻⁸ M) (E2(+): C, D, G and H).](image-url)
report. However, immunoreactive signals were not observed in untransfected 293T cells with anti-ERβ antibody in immunocytochemistry (data not shown). Because these bands were not detected in the nuclear extracts of rat tissues, they did not affect the results of the immunohistochemistry. Two in-frame ATG codons are located in the rat ERβ cDNA sequence (Kuiper et al. 1996). The doublet bands observed in 293T cell transfected with rat ERβ expression vector were probably derived from the usage of both ATG codons for initiation of protein synthesis. The analysis of in vitro translation of rat ERβ also revealed doublet bands (Kuiper et al. 1996). The size of the observed bands in the ovary, uterus and prostate corresponded to that of the lower band detected in 293T cells transfected with the ERβ expression vector. Therefore, we reasoned that the second ATG might be used preferentially in these tissues. The shorter bands detected in 293T cells transfected with rat ERβ expression vector may be derived from some degradation process or alternative usage of ATG codon, since these bands were not detected with anti-ERβ antibody absorbed with synthesized peptide. The 60 kDa band detected in tissues in Western blot analysis using rat ERβ antibody may be the product of alternative usage of ATG or splicing variant.

The ERα immunoreactivity was detected by Western blot analysis using 293T cells transfected with rat ERα expression vector and the nuclear extracts of rat ovary and uterus. No signal for rat ERα was observed in rat prostate and testis. It has been documented that the mRNA of rat ERα was detected in rat prostate by RT-PCR (Kuiper et al. 1997), which suggested that the amount of ERα protein in rat prostate might be too small to detect under our experimental conditions. The shorter bands

![Figure 3. Immunohistochemical localization of ERα and ERβ in rat uterus. ERα was immunostained with anti-ERα antibody in luminal epithelium cells (A) and glandular epithelium cells (B). ERβ was not immunostained with anti-ERβ antibody in luminal epithelium cells (D), but was detected in glandular epithelium cells (E). No immunoreactivity was detected with anti-ERα antibody blocked by synthesized peptide (C), with anti-ERβ antibody blocked by synthesized peptide (F) and with preimmune serum (G and H). le, luminal epithelium; ge, glandular epithelium.](image-url)
bands (55 kDa and 50 kDa) were found in 293T cells transfected with the rat ERα expression vector. These bands may be derived from the alternative usage of initiation ATG. Based on this assumption, if the 6th and 7th ATG of the rat ERα sequence are used in translation, a 55 kDa and a 48 kDa protein respectively could be produced. However, the possibility that these bands may be derived from some degradation process cannot be ruled out.

The nuclear localization of both rat ERα and ERβ in 293T cells transfected with rat ERα and ERβ expression vectors respectively was shown by immunocytochemistry. The cells not stained by corresponding antibodies but stained by DAPI would be untransfected cells. The nuclear localization of these receptors was also confirmed based on the immunohistochemical observations of rat reproductive organs. Glucocorticoid receptor (GR) shows the ligand-dependent translocation into the nucleus from the cytosol (Yang & DeFranco 1994, Sackey et al. 1996). In contrast, the localization of the immunoreactive products for both ERα and ERβ in transfected 293T cells was confined to the nuclei regardless of the presence of E2, implying that the nuclear localization of these receptors is ligand independent (Welschons et al. 1984). The positive immunohistochemical staining for ERα was observed in the nuclei of glandular and luminal epithelium and myometrium in rat uterus. This result was consistent with previous reports (Zaino et al. 1989, Hild-Petito et al. 1988, Hild-Petito et al. 1992, Koji & Brenner 1993, Orimo et al. 1995, Saunders et al. 1997). The staining of ERβ was detected in the nuclei of glandular epithelium in rat uterus, whereas no staining was detected in luminal epithelium and myometrium. Recently, it was reported that ERβ was immunostained in luminal and glandular epithelium of rat uterus (Saunders et al. 1997). We speculate that the amount of ERβ protein is significantly smaller in luminal epithelium than in glandular epithelium.

In the present study, no staining was observed in rat ovary using our polyclonal anti-ERα antibody. Data from several laboratories, using in situ hybridization or immunohistochemistry, have shown that the ERα localizes in granulosa cells and theca cells in certain species (Kudolo et al. 1984, Iwai et al. 1991, Billiar et al. 1992, Orimo et al. 1995, Byers et al. 1997). Saunders et al. (1997) were unable to demonstrate the presence of ERα in rat granulosa cells by immunohistochemistry. Thus, it appears that ERα protein might be expressed at a low level in certain cells of the ovary, but could not be detected by immunohistochemistry.

Recently, an ERα knockout (ERαKO) mouse line has been generated. The females are infertile, associated with hyperemic ovaries with no detectable corpora lutea and hypoplastic uteri, suggesting a crucial role of ERα in reproductive function (Lubahn et al. 1993). In the present study, ERβ was

**FIGURE 4.** Immunohistochemical localization of ERα and ERβ in the rat ovary. ERα was not immunostained with anti-ERα antibody (A). ERβ was immunolocalized to the nuclei in granulosa cells of follicle (C) with anti-ERβ antibody. No immunoreactivities were detected with anti-ERα antibody blocked by synthesized peptide (B), or with anti-ERβ antibody blocked by synthesized peptide (D).

**FIGURE 5.** The expression of ERβ protein through the estrous cycle in rat ovary by immunocytochemistry. Although ERβ was immunostained at the proestrous (A), metestrous (C) and diestrous (D) stages with anti-ERβ antibody, the immunoreactivity of ERβ was hardly detected at the estrous (B) stage in rat ovary.
present in granulosa cells of ovaries as shown by immunohistochemistry. In a recent study, ERβ was revealed in follicle and corpora lutea in the rat ovary (Saunders et al. 1997). In the present study, ERβ was not detected in corpora lutea. The possibility may exist that the amount of ERβ protein in corpora lutea is too small to be detected in the rat ovary under our conditions. ERα was immunostained in granulosa cells in rat ovary, whereas ERα was not detected, which shows that ERβ exists predominantly in the follicles in ovaries. This is in agreement with a previous report which showed the average ratio of ERβ to ERα mRNA was approximately 5:1 (Couse et al. 1997). Couse et al. (1997) discussed the possibility that ERβ is essential to the early stage of follicle growth and that the interaction between the two ERs is required for the late stage of follicle growth. We demonstrated that ERβ was immunostained at the proestrus, metestrus and diestrous stages, but hardly immunostained at the estrous stage in the rat ovary. It was reported that estrogen binding sites were modulated in the ovary by pituitary gonadotropins (Richards 1975). Recently, ERβ mRNA was shown to be down-regulated by gonadotropins using in situ hybridization (Byers et al. 1997). Our results are in agreement with these studies.

In conclusion, we have shown nuclear localization and differential expression of ERα and ERβ in rat ovary and uterus at the protein level. These antibodies are applicable for identifying the subgroup of ERs and thus provide a tool for the investigation of the functional roles of both ERα and ERβ in estrogen target tissues (Katznenellenbogen & Korach 1997).

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