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Expression of Estrogen Receptor-β Protein in Rodent Ovary

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ABSTRACT

Estrogen is an essential hormone for the LH surge and ovulation. The primary source of estrogen is from ovarian granulosa cells and in rats, estrogen, in turn, increases granulosa cell number and enhances FSH-stimulated gene expression in these cells. Thus, rat granulosa cells both respond to and synthesize estrogen. To further elucidate the mechanisms mediating the actions of estrogen in granulosa cells, we have identified and characterized the estrogen receptor-β (ER-β) subtype in rodent granulosa cells. ER-β protein was localized to the nuclei of rat granulosa cells in preantral and antral follicles by immunocytochemistry, coincident with the location of ER-β messenger RNA (mRNA). Immunoprecipitation and Western blot analysis using ER-β specific antisera demonstrated a protein of approximately 60 kDa in granulosa cells prepared from PMSG-primed immature mice and estrogen-treated immature rats. Extracts from granulosa cells specifically bound an estrogen response element and the complex was recognized by antisera to ER-β. A synthetic steroid estrogen radioligand, [^{125}I]-17α-iodovinyl-11β-methoxyestradiol ([^{125}I]-VME2), bound to cytosolic granulosa cell preparations with high affinity (estimated K_D value of 401 ± 83 pM, and B_{max} value of 102 ± 9 fmol/mg protein). ER-β protein levels rapidly declined following hCG treatment consistent with the reported decrease in binding activity and ER-β mRNA levels by high levels of gonadotropins. Overall, we have demonstrated that 1) ER-β protein is the dominant estrogen receptor subtype present in rodent granulosa cells, 2) this receptor is functional, and 3) it is regulated by ovulatory doses of gonadotropins. Thus, ER-β is likely to be a mediator of estrogen action in rodent granulosa cells during follicular development. (*Endocrinology* **140:** 2581–2591, 1999)

ESTROGEN IS REQUIRED for the events leading to ovulation, including enhanced LHRH activity and the LH surge (1). In rats, in addition to its role in the hypothalamus and pituitary, estrogen also regulates ovarian follicular development by stimulating granulosa cell proliferation, increasing gap junction formation between granulosa cells, and enhancing FSH-stimulated gene expression in granulosa cells (reviewed in Refs. 2 and 3). The augmentation of FSH activity increases steroidogenesis and results in greater progesterone and estradiol synthesis. In reproductive age females, the main site of estrogen synthesis is in ovarian granulosa cells (4). Therefore, in some species, estrogen can have both an autocrine and exocrine role.

Presumably, estrogen exerts its biological effects in granulosa cells through classical nuclear receptors. Earlier reports, using radiolabeled hormone, demonstrated that estradiol was taken up by the ovary (5), and binding sites were observed in rat granulosa cell nuclei (6, 7). Later reports further characterized these estrogen binding sites in the ovary (7–10). These binding sites were believed to be estrogen receptors, which are now referred to as estrogen receptor-α (ER-α). ER-α messenger RNA (mRNA) has been detected in rodent granulosa cells using *in situ* hybridization and RNase protection assays but is also present in residual ovarian tissue (thecal and interstitial) (11, 12). ER-α protein has been identified in granulosa cell nuclear extracts by electrophoretic mobility shift assays (13, 14). In contrast, other studies detected ER-α protein in interstitial and theca cells rather than in granulosa cells (15). The importance of ER-α for reproduction can be observed in female mice lacking ER-α (ERKO). These mice are usually infertile, and the ovaries are blocked in follicular development at the small antral stage [(16–18), reviewed in (19)]. However, it is not known if this block in follicular development is due to a primary deficiency in ovarian follicles or if it is secondary to a deficiency in the hypothalamus or pituitary. Nevertheless, it suggests that ER-α is not required for early follicular development in mice.

With the discovery of a second estrogen receptor, ER-β (10, 20), it is possible that some of the estrogen effects in the ovary are mediated through this receptor. ER-β was initially identified in the rat prostate but found to be highly expressed in the rodent ovary (10). The ligand binding domain (LBD) of ER-β is only 55% homologous to the LBD of ER-α (10), suggesting that specific receptor subtype ligands might be identified. Many estrogen-like compounds have similar affinities for ER-α and ER-β, although genistein, a phytoestrogen, has a higher affinity for ER-β than for ER-α (21). Additionally, ER-β and ER-α differ in their responses to agonists and antagonist in cell type and promoter-specific manners (22–24).

In the ovary, ER-β mRNA expression can be detected by RT-PCR (21, 25–28), RNase protection assays (11, 26, 29), Northern blot analysis (20, 25, 26, 28, 30) and *in situ* hybridization (10, 26–28). In addition, splice variants of ER-β are observed in ovary RNA (31–34). ER-β mRNA is specifically localized to granulosa cells using *in situ* hybridization and

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absent (12) or weakly present in interstitial cells (10, 26–28). Using this method, ER-β mRNA was not detected in primordial follicles or oocytes (10), and expression was very low in corpora lutea (11, 27). ER-β mRNA was demonstrated in granulosa cells from ER-α deficient ERKO mice (29). Recent evidence suggests that ER-β mRNA expression in granulosa cells is regulated by the hypothalamic-pituitary axis. Rat ER-β mRNA levels in granulosa cells are relatively constant during follicular development (11, 28) but dramatically decline after high doses of gonadotropins, *e.g.* the LH surge (28) or after treatment with high levels of hCG (11, 28).

Despite the abundance of information on ER-β mRNA, there is little characterization of the ER-β protein in any cell type. Recent reports demonstrate ER-β protein in rat granulosa cells by electrophoretic mobility shift assay (35) and by immunocytochemistry (15). In this paper, we demonstrate the presence of ER-β protein in rodent granulosa cells and its hormonal regulation by ovulatory doses of gonadotropins. A detailed description of ER-β protein was completed using several molecular and biochemical approaches to enhance our understanding of the nature of this protein. The results suggest that ER-β is likely to be a mediator of estrogen action in rodent granulosa cells during follicular development.

Materials and Methods

Animals

Whole ovaries were isolated from immature female Sprague Dawley rats (day 19–23) (Taconic Farms, Inc., Germantown, NY, or Charles River Laboratories, Inc., Wilmington, MA). Granulosa cells were isolated using a 27 g needle attached to a 1-cc syringe as described previously (36). For nuclear extracts, immature rats were treated with 17β-estradiol (1.5 mg/day) (Sigma Chemical Co., St. Louis, MO) for 3 days starting on day 19 to increase granulosa cell number. To determine the effect of gonadotropins on ER-β protein levels, immature female rats (day 21–22) were injected once with 10 IU PMSG (Sigma Chemical Co.) or saline (vehicle). After 48 h, some of the animals were injected with 10 IU hCG (Sigma Chemical Co.). Every 3 h animals were euthanized (4–6 animals/group), ovaries removed and fixed or granulosa cells isolated and frozen at −80 C. For mouse granulosa cells, immature Balb/c mice (day 21)(Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 5 IU PMSG (Sigma Chemical Co.). The mice were euthanized 2 days later, and the ovaries were removed for isolation of granulosa cells as previously described above. Animals were housed in light-controlled rooms with 12-h light, 12-h dark cycles and given food and water *ad libitum*. All protocols had the approval of the Radnor Animal Care and Use Committee (Wyeth-Ayerst Research, Radnor, PA).

Antisera

Antisera were obtained commercially or prepared in-house. The following commercial rabbit antipeptide antisera and the corresponding rat (10) or human (20) ER-β peptide sequences used to make the antisera are as follows: PC168: rat ER-β 467–485 (Oncogene Research Products division of Calbiochem, Cambridge, MA also known as PA1–310 (Affinity BioReagents, Inc., Golden, CO); 6–629: rat ER-β 54–71 (Upstate Biotechnology, Inc., Lake Placid, NY); no. 91: human ER-β 171–185 (human specific, prepared in-house). Each of these antisera recognizes ER-β and does not recognize ER-α. For the detection of ER-α, the antisera SRA-1000 (StressGen, Victoria, BC, Canada) and MC-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and FMS-ER7 (in house, raised against last 21 aa of rat ER-α) (37) were used.

An additional antiserum against ER-β, RB2.3, was raised in rabbits (polyclonal) using the AF-2 region of rat ER-β (R_{182}- Q_{485}). The protein was expressed from a DNA sequence subcloned into bacterial expression vector pET23b (Novagen, Madison, WI), which contains a histidine tag, and was purified using a His-Bind Resin (Novagen) and preparative SDS-PAGE. Rabbits were immunized with RB2.3 and the antiserum collected, affinity purified using a SulfoLink kit (Pierce Chemical Co., Rockford, IL), dialyzed against PBS containing 15 mM sodium azide and 0.2% BSA, and stored at −70 C in aliquots for future use. The titer of the purified antiserum was determined by Western blot analysis using the recombinant protein. This antiserum did not cross-react with a recombinant protein containing the AF-1 region of ER-β (unpublished observation) or full-length ER-α. RB2.3 recognizes both rat and human ER-β protein.

Antiserum to HA1 (Roche Molecular Biochemicals, Indianapolis, IN) was obtained commercially.

Immunocytochemistry

Rat ovaries were fixed by either perfusion or immersion using 2% paraformadehyde and 2% acrolein buffered with sodium phosphate to pH 7.2. The tissue was held in fixative for 1–2 days, cryoprotected overnight with 20% sucrose, frozen, and stored at −80 C. Sections were cryosectioned at 10 μm, air-dried, and stored at −80 C. Immunocytochemistry was done using the buffers of Slayden and Brenner's (38, 39). The sections were pretreated by incubating with a solution containing PBS, 1% sodium borohybride, 0.1% polyvinylpyrrolidone (PVP) 360 (Sigma Chemical Co.), washed, incubated with a solution containing PBS, 1% H_2O_2, 0.3% Triton X-100, PVP 360; then incubated with a solution containing PBS, 10% normal donkey serum (Chemicon), and 0.1% gelatin. The primary antisera for ER-β (PC 168, Oncogene Research Products or PA1 310, Affinity BioReagents, Inc.) were used at 1:2000 or 5 μg/ml dilution, respectively. The secondary antiserum, donkey antirabbit $F(ab)_2$ fragment conjugated to biotin SP (Jackson Immunoresearch Laboratory, West Grove, PA) was used at a 1:1,000 dilution. The Elite standard ABC kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine (40 mg/ml in Tris, pH 7.6 and 0.005% H_2O_2) were used for avidin peroxidase detection.

Immunoprecipitation

Granulosa cells from PMSG-primed female mice or estradiol-primed female rats were plated in dishes in the presence of DMEM:Ham's F-12 (1:1, Life Technologies, Inc., Gaithersburg, MD), supplemented with 1% FBS (HyClone Laboratories, Inc., Logan, UT), 100 U/ml penicillin, 100 U/ml streptomycin, and 1 mM GlutaMAX-1 (Life Technologies, Inc.). The next day, the cells were rinsed with PBS then incubated with DMEM without L-methionine and L-cystine (Life Technologies, Inc.) supplemented with [^{35}S]-EXPRESS Methionine/Cysteine Protein Labeling Mix (NEN Life Science Products, Boston, MA) (122 μCi/35 mm dish or 300 μCi/100 mm dish), 10 mM HEPES buffer solution (Life Technologies, Inc.), 2 mM L-glutamine (Life Technologies, Inc.), and 1 × penicillin/streptomycin solution (Life Technologies, Inc.) for 6 h at 37 C. Cells were washed three times with cold PBS and lysed in cold RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing protease inhibitors (Roche Molecular Biochemicals). Cell extracts were incubated with normal rabbit serum for 30 min at 4 C with rotation and incubated with protein G Sepharose [50% slurry in buffer B (2 mM Tris, pH 7.5, 0.1% SDS, 0.1% NP-40)] (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4 C with rotation. Extracts were centrifuged for 2 min at 10,000 rpm (4 C) and the supernatant was removed. Precleared extracts were incubated with antiserum for 12 h at 4 C with rotation and incubated with protein G Sepharose for 1 h at 4 C with rotation. Samples were centrifuged for 2 min at 10,000 rpm (4 C) and the precipitates were washed five times in cold buffer C (2 mM Tris-HCl, pH 7.5, 0.1% SDS, 0.1% NP-40, 150 mM NaCl). Proteins were solubilized in 2 × Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and separated under reducing conditions on 10% Tris-Glycine gels (Novex) by electrophoresis. Gels were fixed in 30% methanol, 10% acetic acid for 30 min at room temperature, followed by immersion in Amplify (Amersham Pharmacia Biotech) for 30 min at room temperature. Gels were dried under vacuum at 80 C for 30 min and exposed for 12 h to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −80 C.

Western blot

Granulosa cell nuclear extracts were prepared from immature estradiol-treated rats as described previously (40, 41) and homogenized in

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RIPA buffer. Whole ovary tissue extracts from immature rats (Charles River) and liver extracts from adult rats (Harlan Sprague Dawley, Inc.) were prepared by homogenization in RIPA buffer. Rat ER-α and rat ER-β (485 aa, short form) (10) protein were prepared by *in vitro* translation using the TNT kit (Promega Corp., Madison, WI). Rat ER-β protein, long form, was prepared using the full-length rER-β complementary DNA (cDNA) and the TNT kit (Promega Corp.).

Protein (40–50 μg) from tissue extracts were electrophoresed under reducing conditions in a 4–20% or 10% Tris-Glycine gel (Novex, San Diego, CA) and transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was incubated for 1 h at room temperature in blocking buffer [Dulbecco's PBS (D-PBS), 5% Carnation nonfat dry milk, 0.3% Tween-20]. RB2.3 antiserum was applied at a 1:1000 dilution in PMT buffer (PBS, 3% milk, 0.3% Tween-20) and incubated overnight at 4 C. The membrane was washed (4× for 5 min) in wash buffer (PBS, 0.3% Tween-20) and incubated with horseradish peroxidase (HRP)-linked antirabbit IgGs (from donkey) (Amersham Pharmacia Biotech) at a 1:3000 dilution in PMT for 1 h at room temperature. The membrane was washed again (4 times for 5 min each) in wash buffer and two more times for 10 min each in PBS. The membrane was immersed into SuperSignal Ultra Substrate working solution (Pierce Chemical Co.) for 5 min and exposed to X-Omat AR film (Eastman Kodak Co.).

For the hCG time course experiment, the proteins were detected by chemifluorescence according to the directions of the manufacturer (Amersham Pharmacia Biotech). The gel was transferred to membrane, incubated with 5% blocking agent (provided by Amersham Pharmacia Biotech) (1 h, room temperature), washed, then incubated with RB2.3 antiserum (1 h, room temperature). Next, the membrane was washed, incubated with fluorescein-linked antirabbit antibody (1 h, room temperature), washed, and incubated with antifluorescein alkaline phosphatase conjugate (1 h, room temperature). To detect protein, ECF substrate was added to the membrane (20 min, room temperature). The membrane was dried and then scanned using a 570 nm filter with a Storm Imager (Molecular Dynamics, Inc.). The data from two experiments was quantified using ANOVA and LSD tests. Data are presented as mean ± SEM with significance indicated (*, $P < 0.05$).

Electrophoretic mobility shift assays (EMSA)

All reactions were performed in EMSA buffer (20 mM HEPES, pH 7.8, 80 mM KCl, 10% glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT, 200 μg/ml poly d[I-C], 25 μg/ml denatured salmon sperm DNA) in a final reaction volume of 25 μl. Recombinant human (h) ER-α (HA1 tagged) and hER-β [long = 530 aa, (42)], were derived from a baculovirus (bv) expression system (BAC-TO-BAC, Life Technologies) and prepared in extraction buffer (20 mM HEPES, pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 20 mg/ml aprotinin). Granulosa cell nuclear extracts were prepared from estrogen-primed immature rats as described (40, 41). EMSA buffer was supplemented with the indicated protein source (approximately 0.5 μg bv hER-α, 0.5 μg bv hER-β, or 10 μg granulosa nuclear extract) and preincubated for 15–30 min at room temperature. For standard EMSA assays, the reaction was supplemented with 30,000 cpm of [^{32}P]-dCTP-labeled vitellogenin estrogen response element (vitERE) (43) and allowed to proceed for 1 h. Unlabeled vitERE or progesterone response element (PRE) oligos (100-fold molar excess) were added for the competition experiments.

vitERE:

5′-CCAAAGTC**AGGTCA**CAG**TGACCT**GATCAAAGTTAATGTAACCTCA-3′
3′-TTCAG**TCCAGT**GTC**ACTGGA**CTAGTTTCAATTACATTGGAGTACG-5′

PRE:

5′-AGCAAAGTC**AGAACA**CAG**TGTTCT**GATCAA
TTTCAG**TCTTGT**GTC**ACAAGA**CTAGTTCGA-5′

In the ligand-based EMSA assays, the reaction was supplemented with 1 pmol of unlabeled vitERE and 0.75 μl of [^{125}I]-17α-iodovinyl-11β-methoxyestradiol (E isomer) ([^{125}I]-VME2) (1.67 mCi/ml; 2200Ci/mmol, NEN Life Science Products) and allowed to proceed for 1 h. Supershift analysis of the DNA/protein and DNA/protein/ligand interactions were accomplished by adding approximately 1 μg of the indicated antiserum during the preincubation step. Samples were resolved by PAGE for 1.5–2 h at 4 C in 0.5 × TBE. Gels were dried on Whatman paper and exposed to film with an intensifying screen for 16–48 h at −70 C. For the radiolabeled ligand EMSA, after an initial exposure of the gel, the wells were cut off and the gel reexposed to more easily detect the binding complexes in granulosa cells extracts.

Radioligand binding assays

Harvested granulosa cells from immature, untreated rats were washed in PBS, then pelleted by centrifugation (2,000 × *g*, 5 min). Cells were homogenized (30 sec, setting 3, PT1200 polytron, Kimematica; Switzerland) in binding buffer (10 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4 at 37 C) and centrifuged at 50,000 × *g* for 1 h. The supernatant was collected and used for radioligand binding assays. Protein concentration was determined on cytosolic preparations using the Pierce Chemical Co. BCA protein assay with BSA as the standard (44). The cytosol routinely contained approximately 42 μg protein/reaction.

Binding reactions were prepared in triplicate in 96-well microtiter plates. Cytosol preparation (50 μl) was added to each well followed by 25 μl binding buffer for determining total bound or 1 μM 17-β-estradiol (Sigma Chemical Co.) for determining nonspecific bound. Reactions were initiated by the addition of 25 μl of increasing concentrations of 50–2000 pM [^{125} I]-VME2 (2200 Ci/mmol specific activity; NEN Life Science Products) in estrogen binding buffer for a final reaction volume of 100 μl. The reactions were incubated on an orbital shaker for 2 h (25 C). Ice-cold buffer (100 μl) containing 1% (wt/vol) Norit A-activated carbon (Fisher Scientific, Fair Lawn, NJ) and 0.01% (wt/vol) dextran T500 (Amersham Pharmacia Biotech, Uppsala, Sweden) was added to trap unbound estradiol. The microtiter plates were centrifuged at 2,500 × *g* for 10 min and 150 μl of supernatant was removed from each well. The amount of radioactivity present in each sample was measured using an ICN Micromedic 10-channel γ counter (ICN Micromedic Systems, Huntsville, AL). The amount of specifically bound radioactivity was calculated by subtracting nonspecific counts from total counts after correcting the data for a total sample volume (200 μl).

A three-parameter logistic model with parameters K_D, B_{max}, and slope was fitted to evaluate the 2-site saturation models. If the slope estimate indicated a 1-site model (slope not significantly different than 1), the slope was locked to 1, and the analysis was rerun to provide a linear Rosenthal plot. In contrast, if the slope differed significantly from 1, a curvilinear plot was generated, and the 2-site saturation model was run to determine the binding parameters of each binding site. Using this procedure, we were able to demonstrate that a one-site model was suited

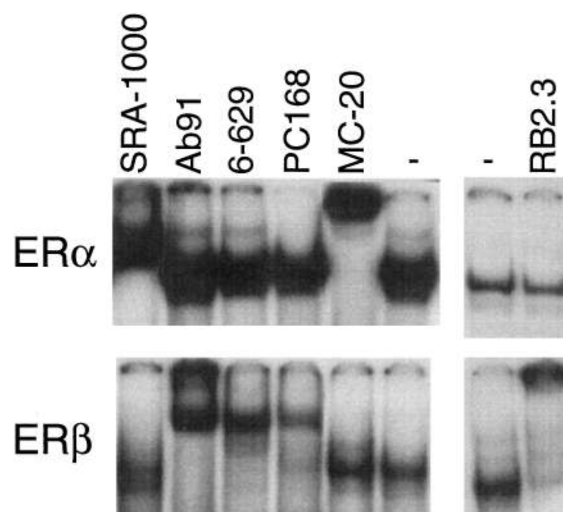

FIG. 1. Specificity of ER antisera. ER antisera were incubated with an ERE and baculovirus-expressed hER-α or hER-β and evaluated using an electrophoretic mobility shift assay. Antisera SRA-1000 and MC-20 specifically recognized ER-α but not ER-β protein, whereas antisera Ab91, 6–629, PC168, and RB2.3 specifically recognized ER-β but not ER-α protein.

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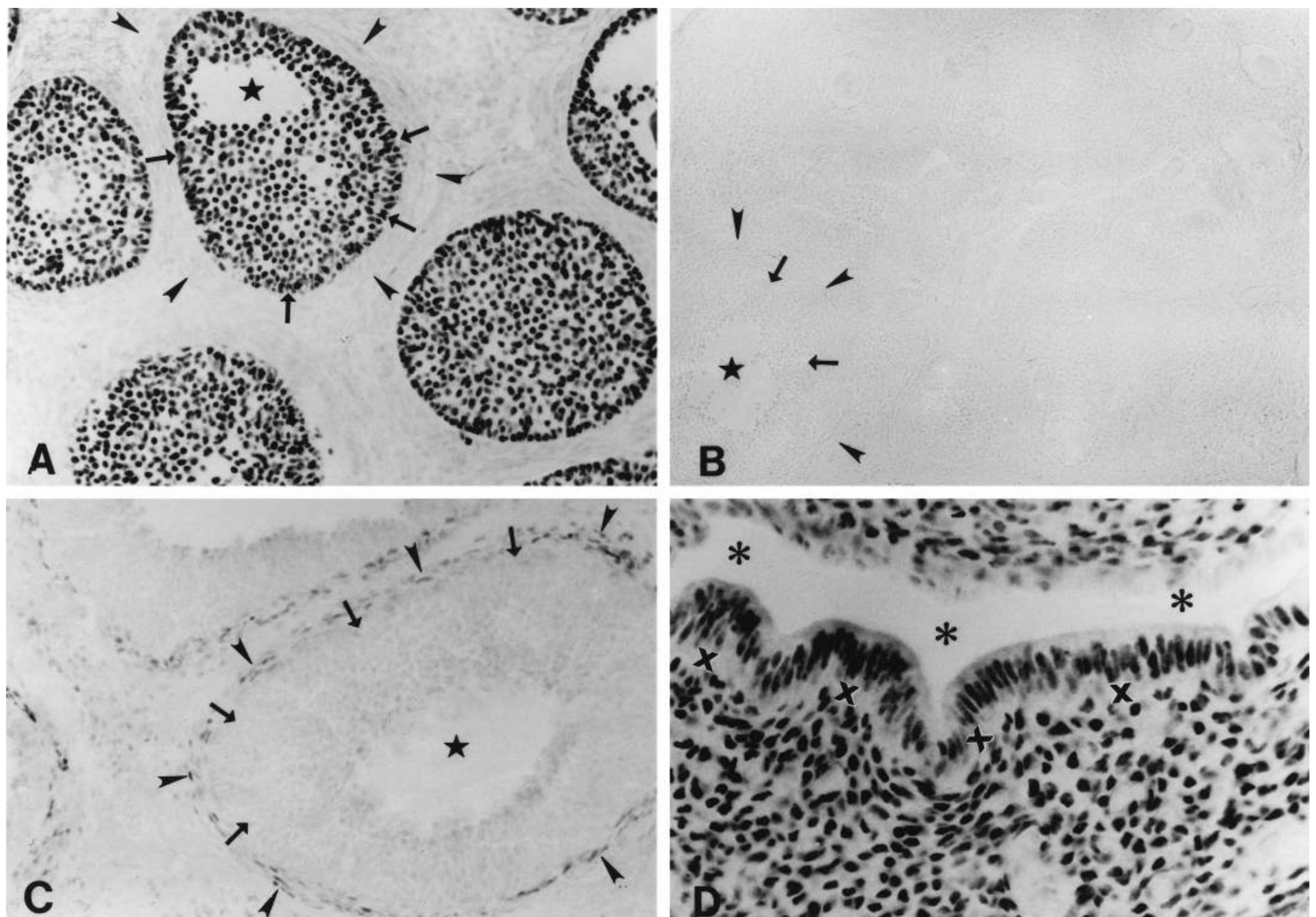

FIG. 2. Detection of ER-α and ER-β protein by immunocytochemistry in the rat ovary. A, Photomicrograph of a rat ovary showing ER-β immunoreactivity in granulosa cells (*arrows*) but not theca cells (*arrowhead*) using the ER-β specific antiserum PC 168. B, As a negative control, no staining was observed in ovary sections incubated with normal donkey serum. C, ER-α staining was observed in theca (*arrowheads*) and interstitial cells but not granulosa cells (arrows) in the rat ovary using the ER-α specific antiserum FMS-ER7. D, As a positive control, strong immunostaining was detected in the uterus with the same ER-α antiserum. *, lumen; X, border between epithelial and stroma tissue. In the ovary sections, *star* indicates the antrum of a follicle. Magnification, A and C, ×250; B, ×125; D ×500.

for our data. The customized JMP applications were developed by Biometrics Research (Wyeth-Ayerst Laboratories, Inc., Princeton, NJ).

Results

Characterization of antisera

The specificity of the antisera used in this paper were tested by EMSA using baculovirus expressed hER-α and hER-β protein. As shown in Fig. 1, antisera SRA-1000, and MC-20 specifically recognized ER-α but not ER-β protein. In contrast, Ab91, 6–629, PC168, and RB2.3 specifically recognized ER-β but not ER-α protein.

Detection of ER-β protein by immunohistochemistry

To identify the localization of ER-β protein, tissue sections of ovary from immature rats were incubated with ER-β antiserum PC 168. Within the ovary, ER-β-immunoreactive cells were seen in granulosa cells of preantral and antral follicles (Fig. 2 A and B). No staining was observed in the interstitium or theca cells. In contrast, ER-α immunoreactivity was detected in theca and some interstitial cells using the ER-α specific antiserum FMS-ER7 (Fig. 2, C and D). The same ER-α antiserum strongly stained cells in uterine tissue. As a negative control, normal donkey serum did not detect any specific staining in the ovary.

Immunoprecipitation of ER-β from rodent granulosa cells

To characterize the size of rat ER-β protein, an immunoprecipitation assay was performed. Granulosa cells from PMSG-primed, immature mice or estrogen-treated, immature rats were incubated with [^{35}S]-Met/Cys containing media, then lysed and incubated with an ER-β specific antiserum (RB2.3) or normal rabbit serum (NRS). Results from mouse granulosa cells indicated that a protein of approximately 60 kDa was detected using the RB2.3 antiserum (Fig. 3, *left panel*). No band was seen with NRS. Using rat granulosa cells, a protein of approximately 60 kDa was observed using RB2.3 but not NRS or rabbit IgG (Fig. 3, *right panel*). This experiment was performed in triplicate and a representative result is shown in Fig. 3.

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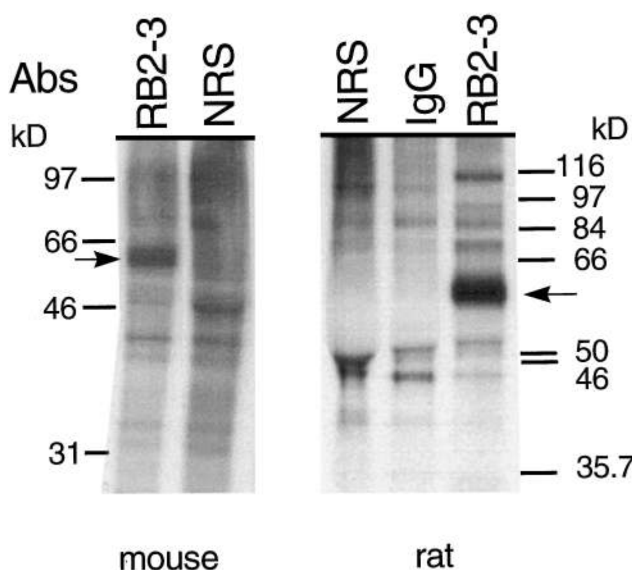

FIG. 3. Immunoprecipitation of ER-β protein from mouse and rat primary granulosa cells. ER-β protein was immunoprecipitated from immature mouse (*left*) or rat (*right*) granulosa cell proteins labeled with ^{35}S-Met/Cys using an ER-β specific antiserum (RB2.3) but not normal rabbit serum (NRS) or IgG proteins. The precipitated proteins were fractionated by SDS-PAGE and revealed by autoradiography. The results are representative of three experiments.

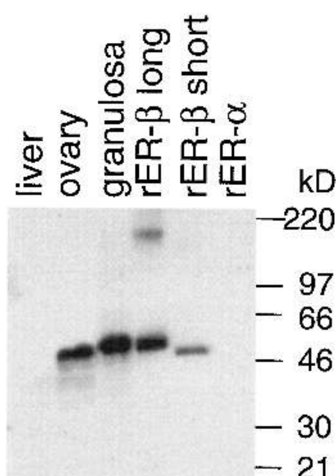

FIG. 4. Detection of ER-β by Western blot analysis. Whole cell extracts from rat granulosa cells, rat ovary or rat liver were separated by SDS-PAGE and transferred to a charged membrane. The blots were incubated with ER-β specific antiserum RB2.3 and the signal detected. *In vitro* transcribed and translated rat ER-α and ER-β short (485 aa), ER-β long (549 aa) served as negative and positive controls, respectively. *Arrow* points to ER-β protein detected in granulosa cells. The results are representative of triplicate experiments.

Detection of ER-β protein by Western blot analysis

Western blot analysis was performed to verify the immunoprecipitation data noted above. Granulosa whole cell extracts and ovary whole tissue extracts from immature rats, and adult rat liver were used to screen for ER-β protein. As controls, *in vitro* translated rat ER-β (short form), rat ER-β (long), and rat ER-α were included. Using peptide-purified antiserum RB2.3, a band of approximately 60 kDa was observed in the granulosa cell lane (Fig. 4). The data shown is typical of the results from three experiments.

Detection of ER-β protein by EMSA

Electrophoretic mobility shift assays provided an additional level of specificity for detecting ER-β protein. The protein must be able to bind a sequence-specific DNA probe and can be further verified by supershifting with antisera. Using granulosa cell nuclear extracts from estrogen-treated immature rats, several protein complexes formed with a consensus ERE DNA probe (Fig. 5A, lane 1, *arrows*). The binding was competed for by an excess of an unlabeled ERE but not a PRE oligomer (lanes 6 and 7). The complexes were supershifted by the presence of ER-β antiserums PC 168, 6–629, and RB2.3 but not by SRA-1000, an ER-α specific antiserum (lanes 2–5). The slower mobility of the RB2.3 complex is likely due to multiple antibodies binding to the protein because RB2.3 is an antiserum directed against the AF-2 domain of rat ER-β, whereas PC168 and 6–629 are antisera directed against peptide sequences. As additional controls, bv hER-β (long form) was supershifted by antiserum 91, which is specifically directed against human ER-β (lanes 9, 10), and by antiserum 6–629 (data not shown and Fig. 4B, lane 6). Baculovirus-expressed hER-α was supershifted by antiserum SRA-1000, which is specific for ER-α (lanes 11 and 12). Interestingly, the ERE oligomer complex formed with granulosa cell proteins was larger than that formed with bv hER-β (long) protein and the ERE oligo (compare lanes 1 to 8). Although ER-α protein was not detected in granulosa cells in this autoradiograph, it was detected in granulosa cells using another ER-α antiserum (MC-20) and a longer exposure (data not shown).

To further characterize the DNA-protein binding complex observed, EMSAs were performed in the presence of a radiolabeled ligand and unlabeled ERE. [^{125}I]-VME2 specifically recognizes estrogen binding sites *in vivo* (45, 46) and binds to both ER-α and ER-β (unpublished observation). The labeled ligand, in the presence or absence of ERE oligomer, migrated near the top of the gel in the absence of protein (data not shown). The labeled ligand bound to bv hER-α or bv hER-β complexed with ERE oligomer, thus detecting the shifted complex (Fig. 5B, lanes 1 and 5). The bv hER-α complex was supershifted with SRA-1000, an ER-α-specific antiserum (lane 3), but not with 6–629, an ER-β-specific antiserum (lane 2). Because bv hER-α contains the HA1 epitope, antiserum to HA1 also supershifted the bv ER-α-DNA binding complex. Alternatively, the ER-β complex was supershifted with 6–629 and 91, both hER-β specific antisera (lanes 6 and 8), but not with SRA-1000, an ER-α-specific antiserum (lane 7). Using granulosa cell nuclear extracts, the labeled ligand detected a protein-ERE complex (lane 9), which was supershifted by 6–629 but not SRA-1000.

Specific estradiol binding sites in rat granulosa cells

To characterize the I-VME binding sites in granulosa cells, cytosol extracts from immature, unprimed rat granulosa cells were incubated with increasing concentrations of

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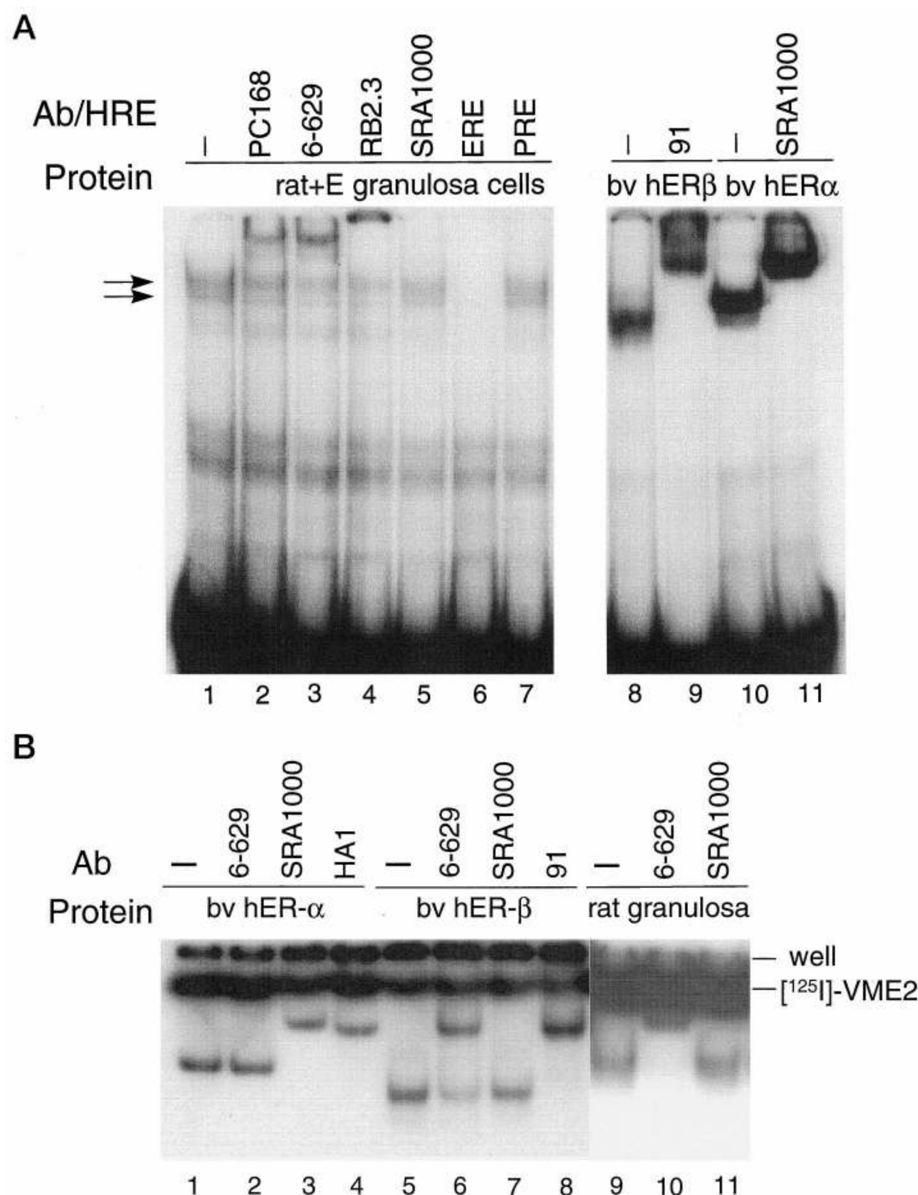

FIG. 5. EMSA for detecting ER-β protein in rat granulosa cells. A, Nuclear extracts from estrogen-primed immature rat granulosa cells formed binding complexes when incubated with [^{32}P]-ERE (lane 1, *arrows*). These complexes were supershifted by the addition of ER-β antisera PC168, 6–629, RB2.3 (lanes 2–4) but not ER-α antiserum SRA-1000 (lane 5). DNA binding specificity was determined by competing with the unlabeled hormone response elements (HRE) ERE (lane 6) or PRE (lane 7) oligomer. As positive controls, bv hER-β was supershifted by ER-β antisera 91 (lanes 8 and 9) and 6–629 (data not shown) and bv hER-α was supershifted by ER-α antiserum SRA-1000 (lanes 11 and 12). The results are representative of three experiments. B, In the presence of an unlabeled ERE, [^{125}I]-VME2 bound bv hER-α (lane 1) or bv hER-β (lane 5). The [^{125}I]-VME2/bv ER-α/ERE complex was supershifted by adding an ER-α antiserum, SRA-1000 (lane 3) or antiserum to the HA1 portion of the bv hER-α fusion protein (lane 4) but not by antiserum to ER-β (lane 2). The [^{125}I]-VME2/bv ER-β/ERE complex was supershifted by antisera to ER-β, 6–629 and 91 (lanes 6 and 8) but not by antiserum to ER-α, SRA-1000 (lane 7). Granulosa cell nuclear extracts from estrogen treated rats bound [^{125}I]-VME2 in the presence of unlabeled ERE (lane 9), and this complex was supershifted by the addition of ER-β antiserum 6–629 (lane 10) but not ER-α antiserum SRA-1000 (lane 11). The portion of the figure containing the granulosa cell extract was exposed longer than the rest of the figure. The results are representative of two experiments.

[^{125}I]-VME2 in the presence or absence of 17β-estradiol. Specific binding increased as the concentration of [^{125}I]-VME2 increased (Fig. 6). A Scatchard analysis revealed a linear relationship between Bound/Free and Bound indicating a single class of receptors with a weighted estimated K_D value of 401 ± 83 pM and a B_{max} value of 102 ± 9 fmol/mg protein (Fig. 6, *inset*).

Hormonal regulation of ER-β protein expression

ER-β mRNA is expressed at a relatively constant level during follicular development but is rapidly down-regulated by high doses of LH or hCG (11, 28). To determine the hormonal regulation of ER-β protein, immature female rats were treated with PMSG for 48 h then hCG for 0–24 h.

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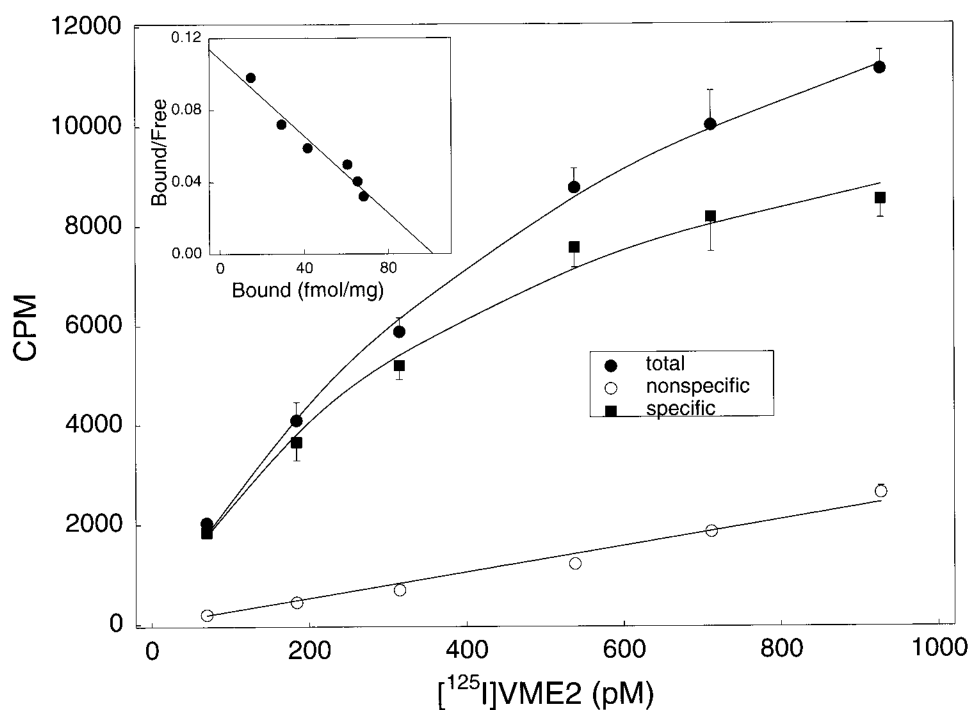

FIG. 6. Saturation binding of [^{125}I]-VME2 to cytosol extracts from immature rat granulosa cells. Varying concentrations of radioligand were incubated with cytosol for 2 h at room temperature. Total (*solid circles*), nonspecific (*open circles*), and specific (*solid boxes*) binding were estimated as indicated in *Materials and Methods*. Line estimation for the saturation isotherms were generated by the SigmaPlot (Jandel Scientific, San Rafael, CA) curve fitting program using hyberbolic, single rectangular 2 parameter fitting. *Inset*, Saturation transformation generated by JMP analysis revealed a K_D value of 401 pM, a B_{max} value of 102 fmol/mg protein and slope value of 1. Data are mean values of triplicate determinations from one experiment that is representative of two experiments.

Immunocytochemistry of ovaries from PMSG and PMSG followed by hCG treated animals are shown in Fig. 7. ER-β protein was demonstrated in granulosa cells of preantral, small antral, and large antral follicles from vehicle-treated and PMSG-treated rats. There was little change in staining 3 h after hCG. In contrast, 9–12 h after hCG, there was a dramatic decrease in ER-β immunoreactivity in large ovulatory follicles but not small antral or preantral follicles. After 24 h, ER-β staining was still observed in small follicles but not in newly formed corpora lutea.

In addition, granulosa cells were isolated from the similarly treated animals, and the amount of ER-β protein present was determined by Western blot using a fluorescence detection system. The results from two experiments were quantified and the mean values with corresponding SEM is shown in Fig. 8. There was no statistical difference in ER-β protein levels in granulosa cells from PMSG- compared with vehicle-treated animals. Statistical analysis revealed that ER-β protein was reduced 9–24 h following hCG treatment compared with PMSG, vehicle, or control values (*, $P < 0.05$).

Discussion

Presented herein is a rigorous characterization of ER-β protein in the rodent ovary. ER-β protein was detected in rat granulosa cells of preantral and antral follicles by immunocytochemistry, which confirms recent work by others (15, 47) and correlates with the location of ER-β mRNA by *in situ* hybridization (10, 28). The lack or low abundance of ER-β protein in other cell types in the ovary indicates that granulosa cells are the primary cells in the rat ovary which synthesize ER-β. Because we and others (11, 15) have demonstrated that ER-α mRNA and protein are preferentially localized in theca and interstitial cells with little or no detection in granulosa cells, the response of granulosa cells to estrogens is likely to be primarily through ER-β rather than ER-α.

The size of the rat ER-β protein produced *in vivo* has not been previously demonstrated and the predicted size has been an issue. The original description of ER-β predicted a protein of 485 aa (~54.2 kDa) (10) although more recent data (42, 48) suggests that the rat ER-β sequence was incomplete. The predicted rat protein, could be either 530 aa [~59.2 kDa, similar to the human long form (42)] or 549 aa (~61 kDa, longest open reading frame with a stop codon before the first methionine). Immunoprecipitations and Western blot analyses with an antiserum directed against the rat ER-β AF-2 domain, RB2.3, revealed a single protein of approximately 60 kDa for ER-β in PMSG-primed mouse and ± E2-primed rat granulosa cells.

The presence of ER-β protein in rodent granulosa cells was further confirmed using electrophoretic mobility shift assays. Several different ER-β antisera supershifted a rat granulosa nuclear extract-ERE binding complex. Detection of a supershifted complex with ER-α antisera depended on the antiserum used and required a much longer exposure, suggesting that the amount of ER-α protein in granulosa cells is quite low. These results are consistent with those recently reported by Clemens *et al.* (35). An additional level of specificity was examined by a ligand based EMSA with the synthetic estrogen [^{125}I]-VME2. [^{125}I]-VME2 has been shown to accumulate in the ovary when injected into rats (45), bind cells containing ER-α and ER-β *in vivo* (49), and, in data presented in this paper, bind both ER-α and ER-β in a ligand-based EMSA. In comparison, 16α-[^{125}I]iodo-E2 has a higher binding affinity for ER-α than for ER-β (21). Nuclear extracts from rat granulosa cells specifically bound to an ERE, forming a complex that bound [^{125}I]-VME2, and the complex was entirely supershifted by an antiserum specific to ER-β. Furthermore,

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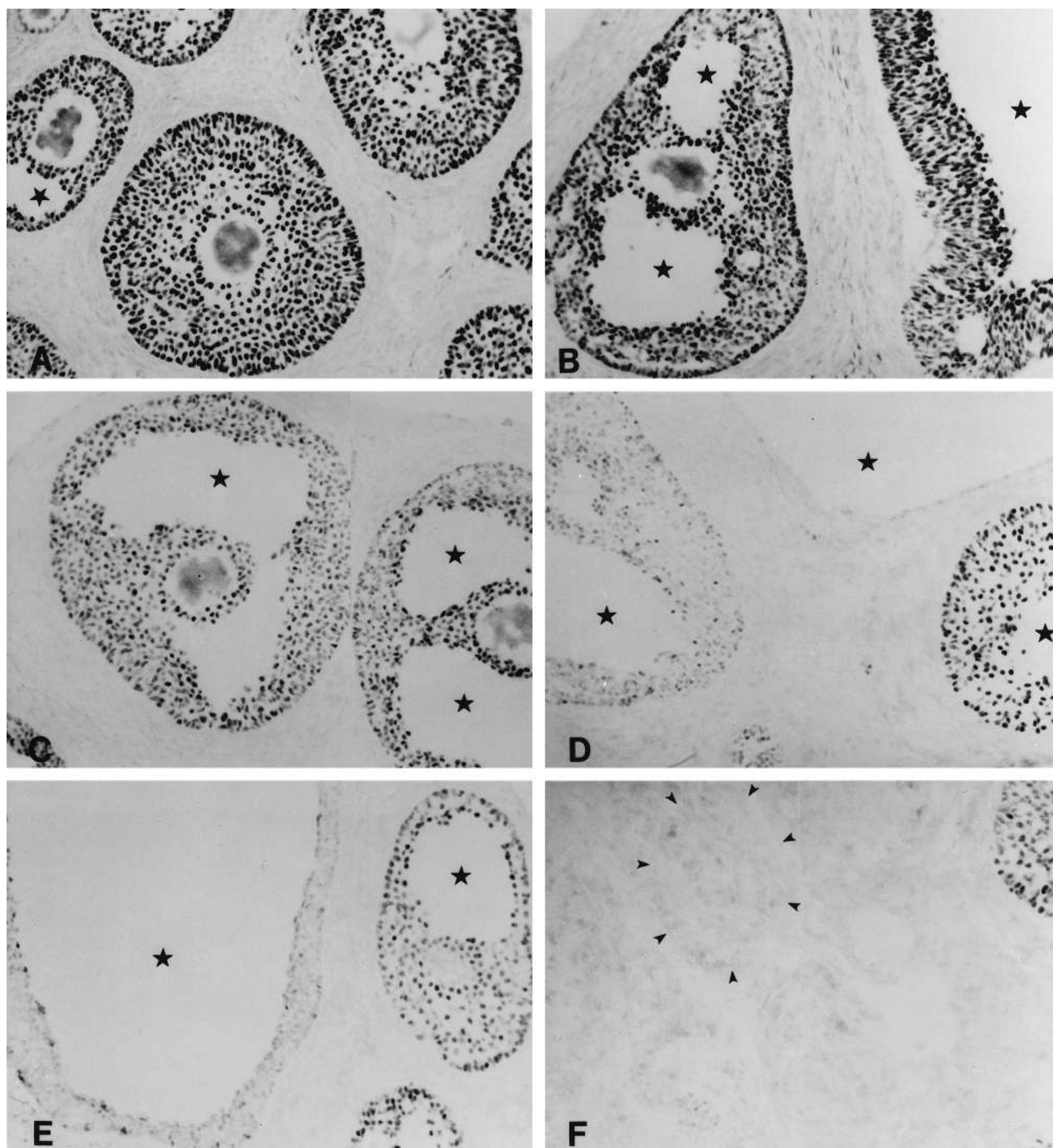

FIG. 7. Effect of hCG on ER-β protein expression using immunocytochemistry. Ovary sections from rats were incubated with an ER-β antiserum (PC 168). ER-β protein was detected in granulosa cells of small and large antral follicles in vehicle-treated (Fig. 7A) and PMSG-treated (B) animals. *Star* indicates the location of antrum in antral follicles. PMSG-treated rats were injected with an ovulatory dose of hCG and ovaries isolated after 3 h (C), 9 h (D), 12 h (E), or 24 h (F). The expression of ER-β protein in granulosa cells 9 h after hCG was reduced in large antral follicles (*left*, D), greatly reduced in Graffian follicles (*center*, D) but did not change in small antral (*right*, D) follicles. Similar expression was observed 12 h after hCG administration (E). One day (24 h) after hCG treatment, ER-β expression was not detected in corpora lutea (*small arrowheads*, F) but highly expressed in preantral (*right*, F) and small antral follicles. Magnification: A–F, ×250.

[^{125}I]-VME2 recognizes a single class of high affinity binding sites in granulosa cells (K_d = 0.4 nM and B_{max} = 102 fmol/mg protein). The binding data were similar to that previously seen with [^{3}H]-estrogen for rat ovary or granulosa cell extracts (7–10). Thus, the present radioligand studies are consistent with those from over 20 yr ago using rat granulosa cells (6). Data presented here suggests that this binding activity is ER-β and identifies ER-β as the dominant estrogen binding species in rat granulosa cells.

ER-β mRNA in rat granulosa cells has been shown to be

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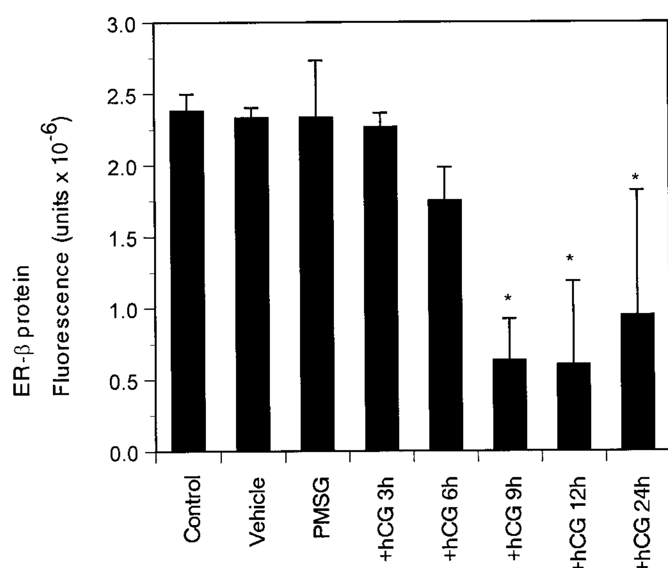

FIG. 8. Regulation of ER-β protein expression by gonadotropins. Granulosa cells were isolated from immature rats treated with PMSG for 48 h, or treated with PMSG for 48 h followed by hCG for 0–24 h (4–6 rats per treatment group). Additional granulosa cells were isolated from 4–6 immature rats 48 h after injection with vehicle (V) or from 4–6 animals at the time of the vehicle or PMSG injections (control). Total protein extracts were prepared and assayed for ER-β by Western blot assay using an ER-β specific antiserum, RB2.3 and chemifluorescence detection system. Baculovirus expressed hER-β served as a positive control. The data were evaluated using the Storm Imager. The results from two experiments were statistically analyzed using ANOVA and the mean ± SE depicted (*, $P < 0.05$ *vs.* control, vehicle, or PMSG treatment).

hormonally regulated by gonadotropins. Low levels of gonadotropins appear to slightly decrease ER-β mRNA levels but high doses, *e.g.* the LH surge or hCG after PMSG treatment, dramatically reduce ER-β mRNA expression (11, 28). We demonstrate here that ER-β protein is not regulated by low levels of gonadotropins but is down-regulated within 9–24 h by high levels of gonadotropins. Specifically, the expression of ER-β protein is greatly reduced in granulosa cells from large ovulatory follicles but not in granulosa cells from small antral or preantral follicles. Likewise, binding of granulosa cell extracts to an ERE was reduced when the extracts were obtained from rats treated with a high dose of hCG compared with control animals (35) and binding of [^{3}H]-estradiol to granulosa cells was reduced in estrogen- and FSH-primed rats treated with an ovulatory doses of LH (6). These results indicate that ER-β mRNA and protein are coordinately down regulated in granulosa cells.

The change in ER-β levels following the LH surge is similar to that seen for a number of other genes expressed in granulosa cells. For example, within 6 h after a surge of LH or hCG, P450 aromatase and LH receptor mRNAs dramatically decline [reviewed in (50)] and ER-α mRNA levels partially decline (11). In contrast, the expression of progesterone receptor (PR) and prostaglandin synthetase-2 (also known as cyclooxygenase-2) mRNAs are induced following the gonadotropin surge (reviewed in Ref. 50). The effects of high doses of gonadotropins on ER-β expression could be a direct stimulation of transcription or an indirect effect, perhaps through the induction of PR. Regardless, there is a complex change in gene and protein expression patterns as granulosa cells start to differentiate and undergo luteinization. The gene expression pattern would suggest that estrogen, ER-α, and ER-β play a role in maintaining and facilitating follicular development but are not required for luteinization.

The precise role and mechanism of action of estrogen in the rodent ovary is not well understood. There are two well documented effects of estrogen in the rat ovary; concomitant increase in granulosa cell number and ovary wet weight (reviewed in Refs. 2, 4, and 51) and enhancement of FSH-induced gene expression (reviewed in Refs. 2–4). Studies of female mice lacking a functional aromatase gene would suggest that estrogen is not required in mice for follicular recruitment but contributes to growth to the antral stage and is required for follicular development beyond the preovulatory stage and for ovulation (52). The relative contributions of ER-α and ER-β to estrogen responses in the ovary are being elucidated. Female mice that lack ER-α are infertile in the absence of exogenous gonadotropins and follicular development is arrested at the preovulatory stage (16, 17, 53). In female adult mice, ovaries appear hyperemic, and follicles contain few granulosa cells (16, 17, 53). However, because the regulation of gonadotropin synthesis and secretion in the hypothalamus and pituitary is impaired in ER-α −/− female mice, the arrest of follicular development and cyst formation that occurs may result from deficiencies directly within the ovary or indirectly within the hypothalamic-pituitary axis. In contrast, the fertility of female mice that lack ER-β is compromised as demonstrated by a reduced number and size of litters. In the ER-β −/− ovaries, follicles of all stages of development are present, although the presence of more atretic follicles and fewer corpora lutea compared with wild-type ovaries suggest a partial arrest of follicular development and less frequent follicular maturation (54). Furthermore, superovulation of these mice results in an increase in mature follicles, but the number of ovulated oocytes is reduced compared with wild-type animals (54). The reduction in ovulated oocytes could be due to fewer ovulatory follicles or a reduced ability of those follicles to respond to gonadotropins. Therefore, ER-β is necessary for modulating and maintaining follicular health and may also contribute to the responsiveness of granulosa cells to high levels of gonadotropins.

The hypothesis that emerges from the combined localization, regulation, and knockout animal studies is that the ovary requires at least two estrogen receptors, ER-α primarily in theca and interstitial cells, and ER-β primarily in granulosa cells, each of which binds estradiol and differentially activates gene transcription through independent cellular and molecular mechanisms. ER-α is likely involved directly or indirectly in earlier events in follicular development and ER-β likely provides a facilitatory role in later events of follicular development before luteinization.

In this paper, we have demonstrated that ER-β protein expression in the ovary is primarily within granulosa cells and that ER-β is the predominant estrogen receptor in rat granulosa cells, consistent with the data of Sar and Welsch (15). ER-β protein is present in granulosa cells of preantral and antral follicles but the level dramatically declines following the ovulatory surge of gonadotropins. Therefore,

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ER-β likely mediates the estrogen responsiveness of granulosa cells during the follicular phase. The confirmation of ER-β as the mediator of estrogen responses in rodent granulosa cells awaits further analysis of functional studies of granulosa cells from normal and ER-β −/− animals.

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References

1. **Freeman ME** 1994 The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, Ltd., New York, 2:613–658
2. **Hsueh AJ, Adashi EY, Jones PB, Welsh Jr TH** 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 5:76–127
3. **Richards JS** 1994 Hormonal control of gene expression in the ovary. Endocr Rev 15:725–751
4. **Shoham Z, Schachter M** 1996 Estrogen biosynthesis–regulation, action, remote effects, and value of monitoring in ovarian stimulation cycles. Fertil Steril 65:687–701
5. **Stumpf WE** 1969 Nuclear concentration of 3H-estradiol in target tissues. Dry-mount autoradiography of vagina, oviduct, ovary, testis, mammary tumor, liver and adrenal. Endocrinology 85:31–37
6. **Richards JS** 1975 Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins. Endocrinology 97:1174–1184
7. **Kudolo GB, Elder MG, Myatt L** 1984 A novel oestrogen-binding species in rat granulosa cells. J Endocrinol 102:83–91
8. **Kawashima M, Greenwald GS** 1993 Comparison of follicular estrogen receptors in rat, hamster, and pig. Biol Reprod 48:172–179
9. **Saiduddin S, Zassenhaus HP** 1977 Estradiol-17β receptors in the immature rat ovary. Steroids 29:197–213
10. **Kuiper GGJM, Enmark E, Pelto Huikko M, Nilsson S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
11. **Tetsuka M, Milne M, Hillier SG** 1998 Expression of oestrogen receptor isoforms in relation to enzymes of oestrogen synthesis in rat ovary. Mol Cell Endocrinol 141:29–35
12. **Shughrue PJ, Lane MV, Scrimo PJ, Merchenthaler I** 1998 Comparative distribution of estrogen receptor-α (ER-α) and β (ER-β) mRNA in the rat pituitary, gonad, and reproductive tract. Steroids 63:498–504
13. **Hillier SG, Saunders PT, White R, Parker MG** 1989 Oestrogen receptor mRNA and a related RNA transcript in mouse ovaries. J Mol Endocrinol 2:39–45
14. **Li S** 1994 Relationship between cellular DNA synthesis, PCNA expression and sex steroid hormone receptor status in the developing mouse ovary, uterus and oviduct. Histochemistry 102:405–413
15. **Sar M, Welsch F** 1999 Differential expression of estrogen receptor-β and estrogen receptor-α in the rat ovary. Endocrinology 140:963–971
16. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
17. **Korach KS** 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524–1527
18. **Couse JF, Curtis SW, Washburn TF, Eddy EM, Schomberg DW, Korach KS** 1995 Disruption of the mouse oestrogen receptor gene: resulting phenotypes and experimental findings. Biochem Soc Trans 23:929–935
19. **Lindzey J, Korach KS** 1997 Developmental and physiological effects of estrogen receptor gene disruption in mice. Trends Endocrinol Metab 8:137–145
20. **Mosselman S, Polman J, Dijkema R** 1996 ER β: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53
21. **Kuiper G G J M, Carlsson B, Grandian K, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. Endocrinology 138:863–870
22. **Watanabe T, Inoue S, Ogawa S, Ishii Y, Hiroi H, Ikeda K, Orimo A, Muramatsu M** 1997 Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptors α and β. Biochem Biophys Res Commun 236:140–145
23. **Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS** 1997 Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510
24. **Tremblay GB, Tremblay A, Labrie F, Giguere V** 1998 Ligand-independent activation of the estrogen receptors α and β by mutations of a conserved tyrosine can be abolished by antiestrogens. Cancer Res 58:877–881
25. **Brandenberger AW, Tee MK, Jaffe RB** 1998 Estrogen receptor α (ER-α) and β (ER-β) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-β in neoplastic tissues. J Clin Endocrinol Metab 83:1025–1028
26. **Enmark E, Peltohuikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA** 1997 Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82:4258–4265
27. **Telleria CM, Zhong L, Deb S, Srivastava RK, Park KS, Sugino N, Parksarge OK, Gibori G** 1998 Differential expression of the estrogen receptors α and β in the rat corpus luteum of pregnancy—regulation by prolactin and placental lactogens. Endocrinology 139:2432–2442
28. **Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK** 1997 Estrogen receptor-β mRNA expression in rat ovary: down-regulation by gonadotropins. Mol Endocrinol 11:172–182
29. **Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138:4613–4621
30. **Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V** 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365
31. **Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA** 1998 Identification of estrogen receptor β2, a functional variant of estrogen receptor β expressed in normal rat tissues. Endocrinology 139:1082–1092
32. **Maruyama K, Endoh H, Sasakiiwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S, Kawashima H** 1998 A novel isoform of rat estrogen receptor β with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action. Biochem Biophys Res Commun 246:142–147
33. **Chu S, Fuller P J** 1997 Identification of a splice variant of the rat estrogen receptor β gene. Mol Cell Endocrinol 132:195–199
34. **Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH** 1998 Estrogen receptor-β mRNA variants in human and murine tissues. Mol Cell Endocrinol 138:199–203
35. **Clemens JW, Robker RL, Kraus WL, Katzenellenbogen BS, Richards JS** 1998 Hormone induction of progesterone receptor (PR) messenger ribonucleic acid and activation of PR promoter regions in ovarian granulosa cells—evidence for a role of cyclic adenosine 3′,5′-monophosphate but not estradiol. Mol Endocrinol 12:1201–1214
36. **Kurten RC, Richards JS** 1989 An adenosine 3′5′-monophosphate-responsive deoxyribonucleic acid element confers forskolin sensitivity on gene expression by primary rat granulosa cells. Endocrinology 125:1345–1357
37. **Shughrue PJ, Scrimo PJ, Merchenthaler I** 1998 Evidence for the colocalization of estrogen receptor-β mRNA and estrogen receptor-α immunoreactivity in neurons of the rat forebrain. Endocrinology 139:5267–5270
38. **Slayden OD, Hirst JJ, Brenner RM** 1993 Estrogen action in the reproductive tract of rhesus monkeys during antiprogestin treatment. Endocrinology 132:1845–1856
39. **Slayden OD, Koji T, Brenner RM** 1995 Microwave stabilization enhances immunocytochemical detection of estrogen receptor in frozen sections of macaque oviduct. Endocrinology 136:4012–4021
40. **Hedin L, McKnight GS, Lifka J, Durica JM, Richards JS** 1987 Tissue distribution and hormonal regulation of messenger ribonucleic acid for regulatory and catalytic subunits of adenosine 3′,5′-monophosphate-dependent protein kinases during ovarian follicular development and luteinization in the rat. Endocrinology 120:1928–1935
41. **Hattori M, Tugores A, Veloz L, Karin M, Brenner DA** 1990 A simplified method for the preparation of transcriptionally active liver nuclear extracts. DNA Cell Biol 9:777–781
42. **Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y, Muramatsu M** 1998 The complete primary structure of human estrogen receptor β(hER β) and its heterodimerization with ER α *in vivo* and *in vitro*. Biochem Biophys Res Commun 243:122–126
43. **Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai MJ, W. OMB** 1989 Specific binding of estrogen receptor to the estrogen response element. Mol Cell Biol 9:43–49
44. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056–1061
45. **Hughes A, Larson SM, Hanson RN, DeSombre ER** 1997 Uptake and interconversion of the Z and E isomers of 17 α-iodovinyl-11 β-methoxyestradiol in the immature female rat. Steroids 62:244–252
46. **Hughes A, Gatley SJ, DeSombre ER** 1993 Comparison of the distribution of radioiodinated-E-17 α-iodovinyl-11 β-methoxyestradiol and 2-iodo-1,1-bis(4-hydroxyphenyl)-phenylethylene estrogens in the immature female rat. J Nucl Med 34:272–280

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47. **Saunders P T K, Maguire S M, Gaughan J, Millar M R** 1997 Expression of oestrogen receptor β (ER-β) in multiple rat tissues visualised by immunohistochemistry. J Endocrinol 154:R13–R16
48. **Bhat RA, Harnish DC, Stevis PE, Lyttle CR, Komm BS** 1998 A novel human estrogen receptor β: identification and functional analysis of additional N-terminal amino acids. J Steroid Biochem Mol Biol 1–8
49. **Shughrue PJ, Lane MV, Merchanthaler I** 1999 Biologically active estrogen receptor-β: evidence from in vivo autoradiographic studies with estrogen receptor α-knockout mice. Endocrinology 140:2613–2620
50. **Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T, Sirois J** 1995 Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Recent Prog Horm Res 50:223–254
51. **Reilly CM, Cannady WE, Mahesh VB, Stopper VS, De Sevilla LM, Mills TM** 1996 Duration of estrogen exposure prior to follicle-stimulating hormone stimulation is critical to granulosa cell growth and differentiation in rats. Biol Reprod 54:1336–1342
52. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci USA 95:6965–6970
53. **Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW, Smith EP** 1996 Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. Recent Prog Horm Res 51:159–186; discussion 186–158
54. **Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 95:15677–15682

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