Journal of Cellular Biochemistry 82:467–479 (2001)

Subcellular Distribution of Native Estrogen Receptor α and β Isoforms in Rabbit Uterus and Ovary

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Abstract The association of estrogen receptors with non-nuclear/cytoplasmic compartments in target tissues has been documented. However, limited information is available on the distribution of estrogen receptor isoforms, specially with regard to the newly described β isotype. The subcellular localization of estrogen receptor α and β isoforms was investigated in rabbit uterus and ovary. Native α and β subtypes were immunodetected using specific antibodies after subjecting the tissue to fractionation by differential centrifugation. The ovary expressed α and β estrogen receptors in predominant association to cytosolic components. However, in the uterus, a substantial proportion of the total estrogen binding capacity and coexpression of the two isoforms was detected in mitochondria and microsomes. The mitochondrial-enriched subfraction represented an important source of 17β-estradiol binding, where the steroid was recognized in a stereospecific and high affinity manner. The existence of mitochondrial and membrane estrogen binding sites correlated with the presence of estrogen receptor α but mainly with estrogen receptor β proteins. Using macromolecular 17β-estradiol derivatives in Ligand Blot studies, we could confirm that both α and β isoforms were expressed as the major estrogen binding proteins in the uterus, while estrogen receptor α was clearly the dominant isoform in the ovary. Other low molecular weight estrogen receptor α-like proteins were found to represent an independent subpopulation of uterine binding sites, expressed to a lesser extent. This differential cellular partitioning of estrogen receptor α and β forms may contribute to the known diversity of 17β-estradiol effects in target organs. Both estrogen receptor α and β expression levels and cellular localization patterns among tissues, add complexity to the whole estrogen signaling system, in which membrane and mitochondrial events could also be implicated. J. Cell. Biochem. 82: 467–479, 2001.

Key words: estrogen receptor; α and β isoforms; subcellular distribution; uterus; ovary

Estrogens are potent regulators of reproductive functions. The classical model of 17β-estradiol action has been traditionally described to be mediated by cytoplasmic/nuclear partitioning receptor proteins that stimulate gene transcription upon binding to specific DNA sequences [Evans, 1988]. However, there are increasing functional evidences for extra nuclear/cytoplasmic localization of steroid hormone receptors. Several studies showing rapid non-genomic actions of steroids have led to speculate about the existence of cell-surface resident receptor forms [Wehling, 1997; Nemere and Farach-Carson, 1998; Revelli et al., 1998]. Whether there exists a membrane receptor for estrogens is still a matter of controversy, but several reports have documented the presence of estrogen binding proteins localized at the plasma membrane [Pietras and Szego, 1979; Pappas et al., 1995; Nadal et al., 1998]. Independently, the known direct effects of various steroids on mitochondrial gene transcription supports the idea of receptor attachment to the mitochondrial genome [Demonacos et al., 1996]. While initial investigations established that the bulk of extranuclear estrogen binding proteins occurred in high-speed supernatant fractions obtained after homogenization of uterine tissue [Gorski et al., 1968], it was also identified early that estrogen specific binding sites were associated with mitochondrial and microsomal structures [Noteboom and Gorski, 1965].

Grant sponsors: Agencia Nacional de Promoción Científica y Tecnológica; Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina.

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Received 30 January 2001; Accepted 20 March 2001

Until recently, it was assumed that estrogens signaled through a single intracellular receptor species, the classical estrogen receptor (ER) α. However, the cloning of a gene encoding a novel ER isoform referred to as ER β, has introduced a new level of complexity to estrogen mediated mechanisms [Kuiper et al., 1996; Tremblay et al., 1997; Gustafsson, 1999 and references therein]. Both ER subtypes share many functional properties, they can bind 17β-estradiol in a high affinity manner and are able to initiate gene transcription under the control of similar response elements [Cowley et al., 1997; Kuiper and Gustafsson, 1997; Kuiper et al., 1997]. Although the biological significance of the existence of receptor isoforms has not been established, the differences in the transactivation domain displayed by the two ERs suggest a specific cellular performance [Giguere et al., 1998]. While mRNA distribution analysis proved that ER β expression could overlap but not exactly resemble the ER α distribution [Kuiper et al., 1997; Gustafsson, 1999], much attention is being paid on protein expression patterns among different tissues and under distinct hormonal environments. The ER β protein distribution and levels may differ from that predicted by the respective mRNA analysis. The occurrence in target cells of estrogen binding activity in several particulate fractions including mitochondria [Noteboom and Gorski, 1965; Pietras and Szego, 1979; 1980], lysosomes [Hisch and Szego, 1974], plasma membrane [Pietras and Szego, 1979; 1980], and microsomes [Watson and Muldoon, 1985; Craig and Muldoon, 1991; Monje and Boland, 1999] has been previously reported, but subcellular localization of both ER α and β proteins has not been studied before.

The aim of the present study was to establish whether extranuclear estrogen binding proteins from rabbit uterus occurred as such in the cytosol and microsomes as we have recently described [Monje and Boland, 1999], or are associated with some other cellular compartment. The subcellular distribution and expression profiles of ER α and β components in their native state were assessed after tissue homogenization and fractionation by classical differential centrifugation methods. Complementary studies on ER α and β protein expression and localization were also carried out in ovary samples from the same species.

MATERIALS AND METHODS

Materials

[(2,4,6,7-^{3}H(N)]17β-estradiol with a specific activity of 80 -115 Ci/mmol was obtained from New England Nuclear (Chicago, IL). Cell culture media and sera were purchased from Gibco (Grand Island, NY). Nonradioactive steroids, tamoxifen, diethylstilbestrol (DES), 17β-estradiol(6-*o*-carboxy-methyl)oxime: BSA fluorescein isothiocyanate conjugate (E_2–BSA-FITC), BSA-FITC and 17β-estradiol–peroxidase (E_2-P) conjugate (1-2 moles estradiol per mole peroxidase type VI) were from Sigma-Aldrich (St Louis, MO). Hydroxylapatite powder was obtained from BioRad Laboratories (Richmond, CA). Anti-ER α mouse monoclonal antibodies clones AER314, AER308, and TE111.5D11 against transactivation, hinge and ligand binding domains, respectively, were purchased as culture supernatants from NeoMarkers (Fremont, CA). The anti-ER β polyclonal antibody PAI-310 (against C-terminal region; aminoacids 468–485) was obtained from Affinity BioReagents (Golden, CO). Anti-ER β polyclonal antibodies against N-terminal (aminoacids 10–28; Y-19) and C-terminal (aminoacids 439–458; L-20) regions were from Santa Cruz Biotechnology (Santa Cruz, CA). Purified recombinant ER α protein was from PanVera Corporation (Madison, WI). Compound $ICI_{182,780}$ was kindly provided by Zeneca Pharmaceuticals (Cheshire, England). The chemiluminescence blot detection kit (ECL) was supplied by Amersham (Piscataway, NJ). All other reagents were of analytical grade.

Subcellular Fractionation

Uteri from 3-month-old female rabbits were collected fresh and placed in ice-cold saline. Uterine tissue from 10–15 individuals was pooled and processed together for subcellular fractionation. Tissue slices were homogenized under ice in TES buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 20 μg/ml aprotinin) with an Ultraturrax homogenizer (5 ml buffer/g tissue). The homogenate was subsequently centrifuged at 800×*g* for 20 min to obtain the nuclear fraction. The supernatant was centrifuged again at 10,000×*g* for 15 min to isolate a mitochondrial-enriched pellet. The remaining supernatant was centrifuged for 1 h

at 120,000×*g* [Craig and Muldoon, 1991]. The supernatant (cytosolic fraction) was collected and the microsomal pellet was washed by resuspension in TES buffer and centrifugation under the same conditions. Nuclear and mitochondrial pellets were washed once with 20 ml of TES medium prior to re-centrifugation. Ovarian samples were collected in parallel and subjected to the same subcellular fractionation protocol.

A part of the final mitochondrial pellet was fixed for 1h, at 4°C, in 1% glutaraldehyde-250 mM sucrose-50 mM cacodylate buffer (pH 7.4), post-fixed in 2% OsO_4 and embedded in Spurr resin for transmission electron microscopy. Sections of 800 Å-thickness were obtained using a LKB ultramicrotome and contrasted with uranile acetate and Pb-citrate. The remaining part of the mitochondrial pellet was suspended in the isolation medium and used for the other measurements. Alternatively, aliquots were frozen in liquid nitrogen and stored at −70°C until determination of estrogen binding activity.

Analytical Determinations

Protein concentration was measured by the method of Bradford [Bradford, 1976] using bovine serum albumin as standard. Contamination of particulate fractions with cytosolic components was assessed by measuring the activity of the cytosolic marker enzyme glucose 6-phosphate dehydrogenase [Cohen and Rosemeyer, 1975]. The activity of the inner mitochondrial membrane enzyme succinate dehydrogenase was determined by means of a kinetic assay by measuring the changes in optical density (600 nm) produced in presence of 0.2 M succinate and 50 μM 2,6-dichloroindolphenol as a final electron acceptor.

[^{3}H]17β-Estradiol Binding Analysis

The total specific estrogen binding capacity (empty plus occupied ER site content) of the several subcellular fractions was determined by incubating 0.3 mg protein samples in 0.2 ml of TES buffer. The reactions were begun by the addition of 4 nM [^{3}H]17β-estradiol. A 100-fold molar excess of radioinert 17β-estradiol was used for determination of nondisplaceable binding. Specific binding sites were then quantified by subtracting non-specific binding sites from sites bound in presence of [^{3}H]17β-estradiol alone (total binding). In related competition experiments, increasing concentrations of unlabelled estrogen-related compounds were added together with the radioligand to additional paired samples. After 4 h of incubation in an ice bath with vortex stirring at 30 min intervals, free [^{3}H]17β-estradiol was separated by resin adsorption of the ligand-receptor complex using the hydroxylapatite (HAP) technique [Wecksler and Norman, 1979]. Briefly, 200 μl of HAP slurry was added to each tube and the suspension was incubated for 15 min at 4°C. Then, the mixture was centrifuged 3 min at 800 × *g*, and the pellets were washed 3 × with TE buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 2 mM DTT, 0.3 mM PMSF) containing 0.5 % Triton X-100, adjusted to reduce nonspecific binding to basal constant levels. HAP pellets were resuspended with 1 ml absolute ethanol and decanted into scintillation vials. Trapped radioactivity was quantified in toluene-based fluid by liquid scintillation spectrometry.

For saturation analysis, samples of fractions containing mitochondria were exposed to a series of [^{3}H]17β-estradiol concentrations ranging from 0.02 to 10 nM. The steroid affinity constant (K_d) and the maximum number of binding sites (B_{max}), were estimated according to the Scatchard equation using the iterative curve fitting LIGAND program [Munson and Rodbard, 1980].

Immunoblots

Each subcellular fraction was analyzed for its immunoreactive ER α and β content. Protein aliquots were combined with one-fourth of sample buffer (400 mM Tris/HCl pH 6.8, 10 % SDS, 50 % glycerol, 500 mM DTT and 2 μg/ml bromophenol blue), boiled for 5 min and resolved by 8 – 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Fractionated proteins on the gels were then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF). The membranes were blocked for 1 h with 5 % non-fat dry milk in phosphate buffer saline containing 0.1% Tween-20 (PBS-T), and were then incubated (1h at room temperature) with the appropriate dilution of each primary antiserum. The membranes were repeatedly washed with PBS-T prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence western blotting kit was used to visualize the immunoreactive products. For reblotting, the

membranes were stripped with denaturing buffer (62.5 mM Tris/ClH pH 6.8, 2% SDS, 50 mM β-mercaptoethanol) at 55°C for 30 min followed by blocking and incubation with primary and secondary antibodies as above. Relative migration of unknown proteins was determined by comparison with wide range colored protein markers. Chemiluminescence images were scanned using a BioRad GS700 densitometer at a resolution of 300 dpi. Determination of the apparent molecular weight of protein bands was done using the program Sigma Gel (Jandel Scientific).

Ligand Blots

Subcellular fractions were subjected to SDS–PAGE on 10% gels and electrotransferred to PVDF membranes. The membranes were exhaustively washed with PBS-T (at least overnight at 4°C) followed by PBS, to remove SDS from proteins and allow their renaturalization on the membranes. After blocking with 5 % BSA in PBS, the membranes were incubated (1 h at room temperature) with 10^{-6} M of E_2-BSA-FITC dissolved in PBS. Recombinant or uterine cytosolic ~67 kDa ER α were used as positive controls of labeling. Reactive bands were visualized under UV light using a conventional transilluminator and photographs were taken using a yellow-greenish filter. In order to intensify label signaling, an alternative detection system based on E_2-P instead of E_2-BSA-FITC was used. To that end, a ligand blot protocol was employed essentially as described by Luconi et al. [1999]. The PVDF membranes containing denatured proteins were incubated overnight at 4°C (or 1 h at room temperature) in the presence of E_2-P (50–10 nM) dissolved in PBS. After several washings with the same buffer, reactive bands were detected by chemiluminescence. The final concentration of the complexes E_2-BSA-FITC and E_2-P was calculated on the basis of their steroid content given by the manufacturer.

Cell Cultures and Transfections

Syrian Hamster uterine myocytes (SHM cells) were grown in Eagle's Minimun Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin. The SHM cell line was a generous gift from Dr. Kirk Riemer (University of California, San Francisco). The cells were cultured at 37°C in a humidified atmosphere containing 95% air-5% CO_2. Semi-confluent SHM myocytes were transfected using varying amounts (2–5 μg) of the pCMXmERβ expression vector containing the complete cDNA of murine ER β [Tremblay et al., 1997]. Cells were incubated for 3 h with the transfection mixture (Lipofectamin Plus, Life Technologies) in α-MEM devoid of serum. After the addition of fresh media containing 5 % FBS, the cells were allowed to grow for 48 h. The monolayers were scrapped and immunodetection of over-expressed ER β from total homogenates was carried out as previously described.

RESULTS

To assess the subcellular distribution of estrogen binding sites in major subfractions of uterine and ovary tissues, specific binding of tritiated 17β-estradiol was quantified in saturation experiments. Reproducible specific binding activity could be measured in all the subcellular fractions tested. The results of a typical experiment are depicted in Figure 1, which tries to emphasize the presence of estrogen binding sites associated to non-classical particulate sources of ER, i.e. fractions containing mito-

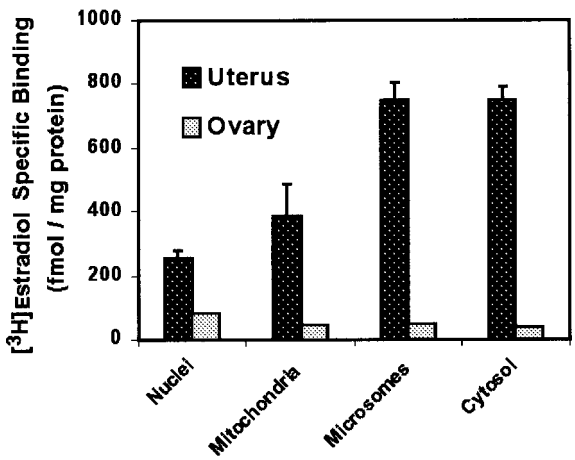

Fig. 1. [^{3}H]17β-estradiol binding capacity of subcellular fractions from rabbit uterus and ovary. In each experiment, estrogen binding site concentration was quantified by saturation binding assays in nuclear, mitochondrial, microsomal and cytosolic subfractions. Equal protein samples were incubated for 4 h in presence of 4 nM [^{3}H]17β-estradiol alone or in combination with 100-fold molar excess of cold 17β-estradiol. The concentration of specific 17β-estradiol binding sites was determined as indicated in Methods. Results are expressed in fmol/mg protein and represent the mean of samples analyzed in triplicate ±SD. A representative ligand binding assay from 4 independent isolation and quantification experiments is depicted.

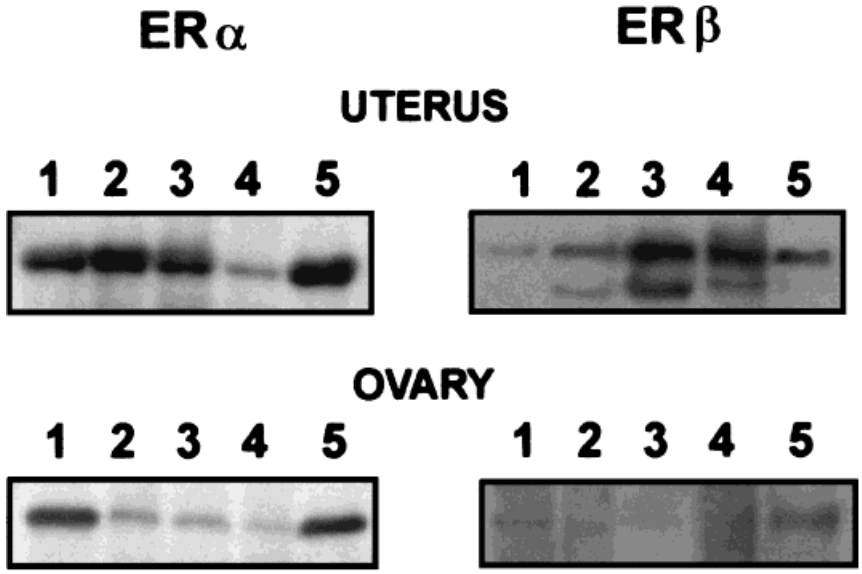

Fig. 2. Subcellular distribution of ER α and β isoforms: comparison of expression profiles in uterus and ovary. Equivalent protein samples (40 μg) were resolved by SDS-PAGE, followed by Western Blot analysis using anti–ER α (AER308) and anti-ER β (Y-19) antibodies. Images developed with anti-ER α antibodies were focused to show the ~67 kDa protein band expected for this isoform. The anti-ER β antibodies labeled one or two main bands of ~45–50 kDa which comigrated in both tissues. Subcellular fractions from uterus and ovary were developed simultaneously in order to obtain comparable relative intensities of immunolabeling. **Lane 1**: total homogenate; **2**: nuclear fraction; **3**: mitochondria; **4**: microsomes; **5**: cytosol.

chondria and microsomes. Cytosolic and nuclear fractions were included as controls on the basis of being the major expected estrogen binding site pools. As a rule, total specific estrogen binding activity resulted markedly lower in ovary than in uterus.

To immunologically correlate [^{3}H]17β-estradiol binding capacity with ER α and/or ER β constituents, each fraction was resolved by SDS–PAGE followed by Western analysis using anti-ER α or anti-ER β specific antibodies (Fig. 2). As expected, high expression levels of the ER α isoform were detected in total homogenates and cytosol of both uterus and ovary. Ovarian subcellular fractions were employed for comparison, specially for experiments on ER β immunolocalization. Previous studies based on mRNA quantification in the female reproductive tract of other mammalian species showed that the ovary is the organ expressing the greatest concentration of ER β transcript [Byers et al., 1997; Couse et al., 1997]. In the uterus, ER β was mainly localized in particulate subfractions, where both mitochondrial-enriched preparations and microsomes represented the most significant sources. This distribution does not coincide with that predicted according to the classical localization of the α isoform, which could be usually recovered in cytosolic fractions. However, estrogen uterine binding data correlates well with the fact that mitochondrial and microsomal preparations could express an important content of ERs, specially the ER β isoform. In the ovary however, this isoform was predominantly associated to cytosolic components (Fig. 2). The expression levels of both ER subtypes in the latter organ were generally lower when compared to samples of uterine origin. Coexpression of ER α and β in rabbit uterus and ovary, as well as colocalization of the two ER known species in uterine particulate fractions, appeared to be a constant feature among independent experiments.

To exclude the possibility of nuclear contamination that would account for ER α and β localization in uterine fractions bearing mitochondrial elements, pellet samples from the latter subfractions were fixed to be visualized by electron microscopy. Neither whole nucleus nor nuclear substructures could be identified in these preparations. The fractions contained mitochondria and single membrane vesicles, probably of lysosomal origin (not shown). The mitochondrial marker enzyme succinate dehydrogenase partitioned as follows (in arbitrary units): 0.0295, 0.045, 0.2025, and 0.0095 among

total homogenate, nuclei, mitochondria and microsomes, respectively, and was not detected in the cytosol. The absence of contaminating cytosolic components within particulate subfractions (mitochondria and microsomes) was confirmed by determining the activity of glucose 6-phosphate dehydrogenase.

In a recent work we have described the presence of ER α-like membrane proteins derived from rabbit uterine microsomal preparations. By means of a selected set of antibodies directed against specific sequences corresponding to the transactivation, hinge and ligand binding domains on the ER α protein, we could detect labeling of low molecular mass proteins apart from the ~67 kDa expected band that corresponds to the intracellular receptor [Monje and Boland, 1999]. The present observations indicate that the ER β isoform could also be attached to the membranes, pointing to the existence of an additional source of estrogen binding activity in these fractions. The antibodies directed against the ER β isoform displayed no cross-reactivity with any of the described immunoreactive ER α subspecies from microsomal uterine membranes (Fig. 3C). These antibodies reacted indeed with a tight doblet/triplet of ~45–50 kDa of apparent molecular mass (Fig. 2). As shown in Figure 3A, immunoblots from SHM uterine cells transfected with an expression vector containing the complete cDNA of murine ER β, expressed the same three reactive bands. The specificity of the immunoreactivity was further evidenced by the fact that the antibodies did not react with COS-1 cells not endowed with ERs; in addition, as

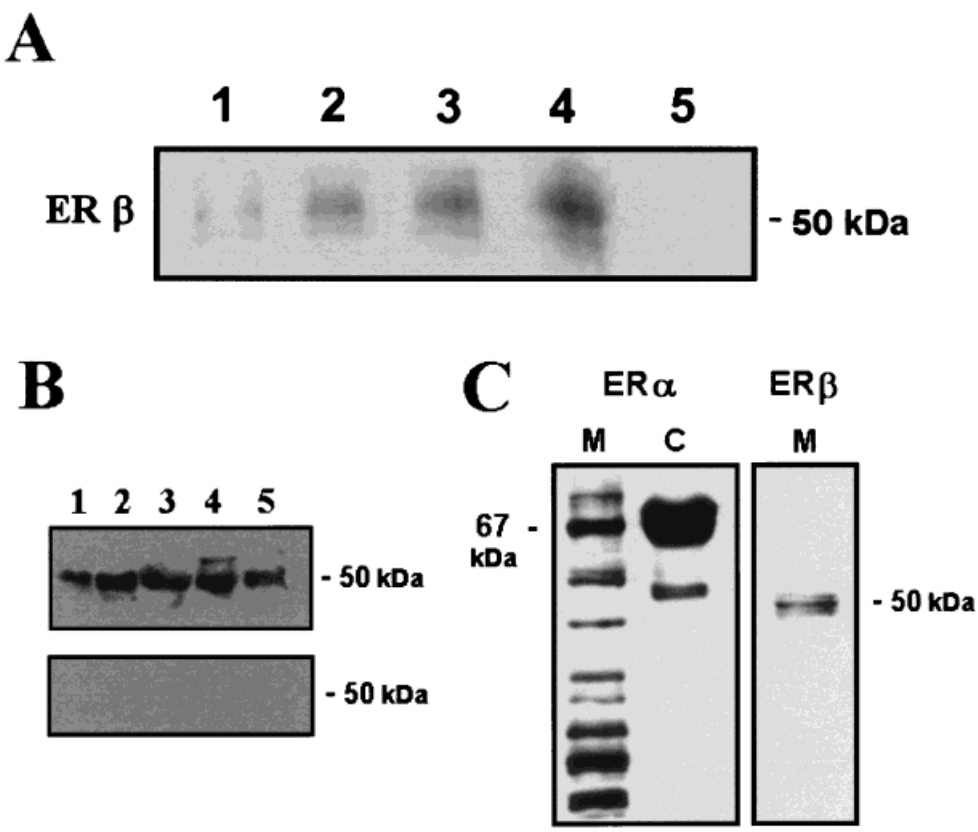

Fig. 3. Control of specificity and evaluation of cross-reactivity of anti-ER β antibody labeling. **A**. Positive and negative controls for anti-ER β recognition. SHM cells were transiently transfected with cDNA for the complete murine ER β. Total homogenates from transfected and non-transfected cells were resolved by SDS-PAGE and immunoblotted with the anti-ER β antibody (PAI-310). **Lane 1:** control (cells not transfected); **2-3-4:** cells transfected with 2, 5 and 5 μg of plasmid DNA; **5:** total homogenate of COS-1 cells (ER-negative). **B**. Negative control for anti-ER β labeling. Uterine fractions were incubated with the anti-ER β L-20 (upper panel) or with secondary peroxidase-conjugated antibodies alone (lower panel). **Lane 1**: homogenate; **2**: nuclei; **3**: mitochondria; **4**: microsomes; **5**: cytosol. **C**. Evaluation of cross-reactivity between anti-ER α and anti-ER β antibodies in uterine fractions. Proteins were resolved on 10% poliacrylamide gels and then immunoblotted with anti-ER α monoclonal antibodies (AER308). Afterwards the membranes were stripped and reblotted with anti-ER β antibodies (L-20). Lane M: uterine microsomes; C: cytosol.

shown in Figure 3B secondary antibodies did not recognize any proteins ranging within the expected molecular size. A similar feature of ER β expression was reported by others [Kuiper et al., 1996; Hiroi et al., 1999] and could be the result of alternative usage of different in-frame initiation codons [Kuiper et al., 1996] or variants generated by differential splicing [Chu and Fuller, 1997]. The finding of close-multiple bands for ER β in uterine preparations, indicates that these ER β-like forms could be expressed in vivo and have physiological relevance.

In an attempt to characterize mitochondrial estrogen binding sites and thus compare them with the previously described microsomal sites, the association of [^{3}H]17β-estradiol to uterine mitochondrial-enriched preparations was analyzed in equilibrium binding experiments. A detailed characterization of the latter estrogen binding sites has not been, to our knowledge, reported elsewhere. As shown in Figure 4A, the specific binding of the hormone was a saturable process with respect to the ligand concentration. The nondisplaceable binding was remarkably low and never exceeded 5 % of total binding over the whole radioligand concentration range used. Saturation data analyzed by linear Scatchard transformation revealed a single high affinity binding component for 17β-estradiol ($K_d = 0.40$ nM) with a maximum binding capacity of ~ 580 fmol/mg protein (Fig. 4B). The relative affinity of mitochondrial estrogen binding sites for a range of compounds was determined by competition binding assays. These studies showed that 17β-estradiol displaced the radioactive ligand with high efficiency compared to the stereoisomer 17α-estradiol (Fig. 4C). The IC_{50} value estimated for 17α-estradiol was about 100 times higher with respect to that of 17β-estradiol. As shown in Figure 4D, mitochondrial 17β-estradiol binding could also be displaced by classical ER ligands such as DES, tamoxifen and ICI182,780. As the latter three are not chemically related to estrogen molecules and have proven to be recognized by both ER α and β isoforms [Kuiper et al., 1997], one may speculate that both isoforms could be independently contributing to the observed binding characteristics.

In order to establish the relative contribution of each ER α and β isoform in subcellular fractions from uterine tissue, we used macromolecular 17β-estradiol derivatives to detect estrogen binding proteins in Ligand Blot experiments. To that end, the proteins of each

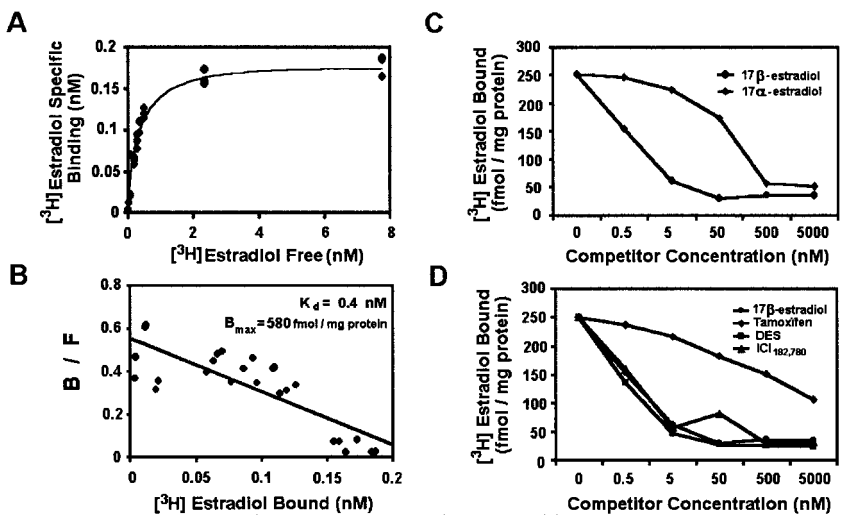

Fig. 4. Saturation and specificity analysis of [^{3}H]17β-estradiol binding to mitochondrial sites from rabbit uterus. **A**. Protein fractions were incubated with increasing concentrations of [^{3}H]17β-estradiol with or without a 100-fold molar excess of unlabelled 17β-estradiol. Incubations were performed for 4 h under ice until separation of unbound steroid by the HAP procedure. Specific radioligand binding (B) was plotted against the concentration of free tritiated hormone in the incubation medium (F). Each point represents a mean value of triplicate determinations from one representative experiment. **B**. Graphical analysis of the same data by the method of Scatchard. Estimated binding parameters at equilibrium are indicated. **C**. Inhibition of radioligand binding by the 17α stereoisomer. **D**. Displacement curves for non-steroidal estrogenic and antiestrogenic compounds. [^{3}H]17-β-estradiol (5 nM) and a range of 0.1-1000 fold-molar excess of cold competitors were incubated simultaneously with 0.3 mg protein for 4 h, at 4°C. Bound radioligand in the absence of competitor was set as total binding. Displacement curves for 17β-estradiol are included as a reference.

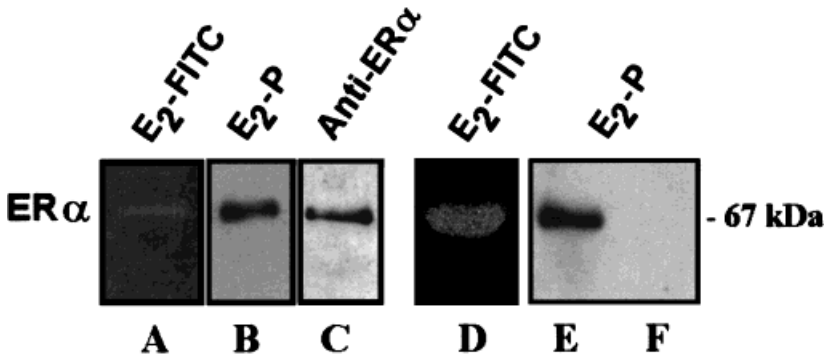

Fig. 5. Identification of ER α from control sources by Ligand Blot analysis. Detection of the ~67 kDa ER α recombinant protein using (**A**) 17β-estradiol-BSA-FITC (E_2-FITC), (**B**) 17β-estradiol-peroxidase (E_2-P) and (**C**) anti-ER α antibody (clone TE111.5D11). Samples of 5 μg (A) and 2.5 μg (B y C) of recombinant purified protein were analyzed as described in Methods. (**D**) and (**E**) 60 μg and 10 μg, respectively, of uterine cytosolic protein were used. (**F**) 60 μg protein of total homogenate of COS-1 (do not express ERs) was tested as a negative control of labeling. Probing transferred membranes with BSA-FITC alone (control) produced no protein staining.

subfraction were resolved by conventional SDS–PAGE followed by electrotransference to PVDF membranes. After renaturalization and blocking of nonspecific sites, the membranes were incubated with the fluorescent estrogen conjugate E_2-BSA-FITC or alternatively with E_2-P. In the first case, the reactive proteins were visualized by direct exposure to UV light. This technique represented an adaptation of the Ligand Blot protocol employing 17β-estradiol-BSA-I^{125} followed by autoradiography, which was reported by Zheng and Ramirez [1997]. By using E_2-P instead of the fluorescent derivative, we found a notorious improvement in the detection sensitivity of reactive proteins, and the problem of FITC emission photobleaching was eliminated. The renaturalization step proved to be an essential requirement to develop estrogen binding reactivity, arguing in favor of the specificity of labeling. Figure 5 shows results obtained with purified recombinant ER α and uterine cytosol as positive controls. Simultaneously, total lysates of COS-1 cells were used as negative controls. Figure 6 (left) shows the complete subcellular localization profile for the major uterine estrogen binding proteins, using E_2-BSA-FITC as ligand. Figure 6 (center) shows the equivalent pattern obtained with E_2-P. It is clearly appreciated the labeling of ~67 and ~45–50 kDa protein bands, which colocalized with the immunological detection of ER α and β isoforms, respectively (Fig. 6 - right).

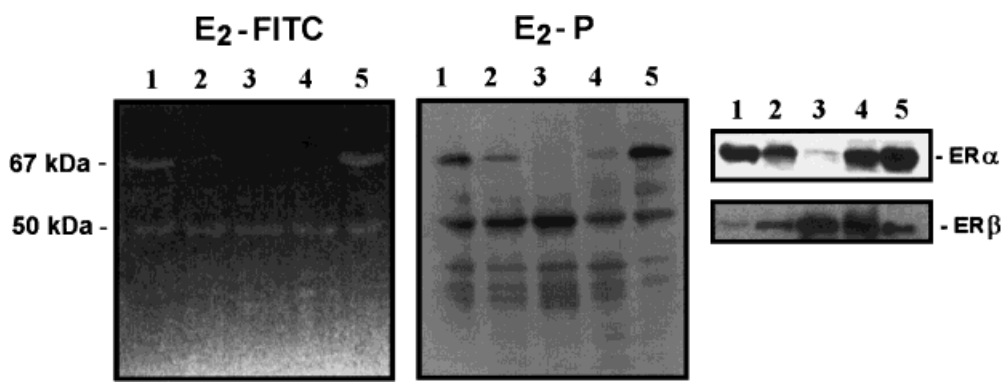

Fig. 6. Subcellular distribution of 17β-estradiol binding proteins from uterus as revealed by Ligand Blot analysis. Subcellular fractions from rabbit uterine tissue were isolated as described in Methods. After SDS-PAGE and transference to PDVF membranes, estrogen binding proteins were labeled with 17β-estradiol-BSA-FITC (E_2-FITC; left) or 17β-estradiol-peroxidase (E_2-P; center) conjugates. Immunoblot patterns using anti-ER α (TE111.5D11) or anti-ER β (Y-19) antibodies are shown for the same fractions (right). **Lane 1**: total homogenate; **2**: nuclei; **3**: mitochondria; **4**: microsomes; **5**: cytosol. For detection with E_2-FITC and E_2-P, 50 μg and 10 μg protein, respectively, were applied per lane.

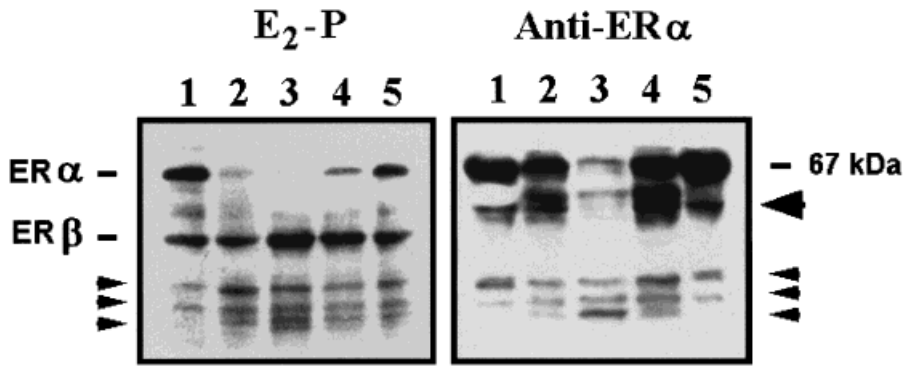

Fig. 7. Detection of low molecular weight estrogen binding proteins from uterine subcellular fractions by Ligand Blot (left) and Western Blot (right) analysis using 17β-estradiol-peroxidase (E_2-P) and anti-ER α antibodies (TE111.5D11), respectively. The expected bands for the wild type ER α and β isoforms are shown. The position of the two main groups of ER α-like immunoreactive proteins of ~60–55 kDa (thick arrow) and ~35–28 kDa (thin arrows), are also depicted. The latter group of proteins was developed simultaneously by both detection procedures. For E_2-P and immunoblot labeling, 10 and 40 μg protein, respectively, were applied per lane. Note that anti-ER α antibodies had no cross-reactivity with ER β protein bands. **Lane 1**: total homogenate; **2**: nuclei; **3**: mitochondria; **4**: microsomes; **5**: cytosol.

Moreover, the intensity of reactive estrogen binding proteins on the blots correlated well with the apparent level of ER α and β in each subcellular fraction. Ligand Blot studies showed that a significant proportion of 17β-estradiol binding proteins could be associated to the expression of ER β in mitochondrial- and membrane-containing fractions, whereas ER α was predominantly recovered in the cytosol. The presence of other bands of weaker intensity than those corresponding to the α and β isoforms were revealed by this technique. Although quantification of absolute levels of ER α- and ER β-like binding proteins depends basically on the degree of renaturalization of the receptor proteins, Ligand Blot experiments allowed us to establish not only the presence of either isoform in a given subfraction, but also estimate their relative contribution to the total estrogen binding. The differential subcellular localization of ER α and β binding proteins found in rabbit uterine tissue represents an original contribution.

From the comparison of estrogen binding and ER α immunoreactive patterns, it is evident that proteins smaller than the expected molecular weight for the wild type ER α, contribute to the binding of estrogen (Fig. 7). The detection of several uterine ER α-like binding proteins of low molecular weight is in agreement with our previous studies [Monje and Boland, 1999]. Proteins of ~55–60 and ~35–28 kDa appeared as major ERα-like bands in membrane subfractions. The same ~55–60 kDa bands were present in the nuclear fractions but absent in the cytosol. Ligand Blot analysis did not reveal the presence of estrogen binding proteins of similar molecular weight, intensity or subcellular distribution pattern. Thus, we speculate that the latter proteins would not correspond to estrogen binders. However, the ~35–28 kDa bands that mainly localized in particulate fractions, could be detected by both anti-ER α antibodies and peroxidase-coupled macromolecular derivatives of 17β-estradiol (Fig. 7). Although we have not identified the molecular nature of these ER α -related small proteins, probably ER α splice variants, we suggest they are indeed capable of binding estrogen. This group of low molecular weight proteins might contribute to the estrogen binding capacity detected in uterine preparations, apart from that originating from the expression of the major wild type isoforms ER α and β.

We also investigated the localization of α and β ERs in subcellular fractions from rabbit ovary (Fig. 8, left and center). Their distribution in this tissue was notoriously different from that observed in the uterus. Immunoreactive levels of both isoforms were considerably lower in ovary, which agrees with the low specific binding of tritiated 17β-estradiol measured by conventional binding assays (Fig. 1). ER α and β partitioned roughly to the same extent among subfractions; both isotypes were principally located in the cytosol. Surprisingly, Ligand Blot studies using E_2-P revealed that the ER α isoform was expressed as the major

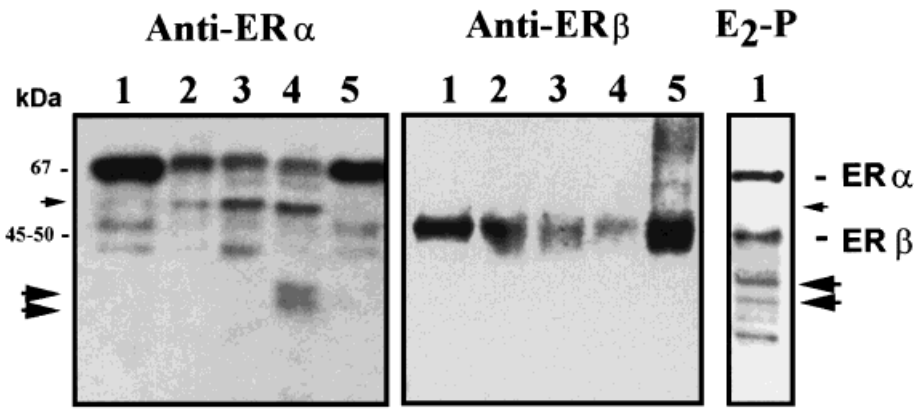

Fig. 8. Expression of ER α and β in ovary: subcellular distribution. Complete subcellular fractions from rabbit ovary were probed using anti-ER α AER 308 (left) and anti-ER β Y-19 (center) antibodies. A Ligand Blot profile using 17β-estradiol-peroxidase (E_2-P) is also included (right). **Lane 1**: total homogenate; **2**: nuclei; **3**: mitochondria; **4**: microsomes; **5**: cytosol. For E_2-P and immunoblot detection, 20 and 40 μg protein, respectively, were applied per lane. Again, anti-ER α antibodies revealed the presence of proteins of ~60–55 kDa (thin arrows) in nuclei, mitochondria and microsomes, which were not detected by Ligand Blot analysis. The position of microsomal proteins of ~32–28 kDa is shown (thick arrows). These two groups of ER α-like proteins comigrated with the homologous uterine counterparts.

estrogen binding protein in rabbit ovary (Fig. 8, right).

DISCUSSION

The expression levels of the ER α and β isoforms and their apparent subcellular localization under native conditions were studied in rabbit uterine and ovarian fractions isolated by standard differential centrifugation procedures. In agreement with previous reports, we obtained further evidences supporting the existence of appreciable quantities of specific [^{3}H]17β-estradiol binding sites concentrated not only in the cytosol and nucleus but also in association with microsomal and mitochondrial membranes. To ascertain the nature of these sites, probing of ER α and ER β was undertaken using specific antibodies with well defined antigenic targets on each isoform. The approach allowed us to immunologically discriminate between ER α and β components in subcellular fractions bearing abundant [^{3}H]17β-estradiol binding capacity. By means of Ligand Blotting assays using macromolecular 17β-estradiol derivatives we could also establish the relative contribution of each isoform to the total detected estrogen binding sites. In the case of microsomal membranes, we have first estimated that ER α-like sites may account for ca. 50% of their estrogen binding activity, by measuring the inhibition of [^{3}H]17β-estradiol binding using an antibody against the steroid binding domain of this isoform [Monje and Boland, 1999].

The demonstration of both estrogen binding sites and anti- ER α/β immunological reactivity in uterine fractions enriched in mitochondria and microsomes, is noteworthy. Our classical description of mitochondrial sites by radioligand binding essays rendered high affinity and specificity for estrogens. These properties are typically associated with true receptor proteins and closely resemble those of the respective microsomal counterparts [Watson and Muldoon, 1985; Craig and Muldoon, 1991; Monje and Boland, 1999]. Results obtained from radioligand binding assays using subcellular fractions derived from uteri have traditionally rendered good adjustments to single apparent estrogen binding sites [Pietras and Szego, 1979; Watson and Muldoon, 1985]. Instead, in view of the present observations, we conclude that these subfractions could be indeed complex mixtures bearing at least two types of ER-like major estrogen binding subpopulations. Scatchard analysis could not dissect into ER α and β binding sites since both receptors are able to bind their cognate ligand with very similar affinities [Kuiper et al., 1997].

Our results are consistent with previous studies based on mRNA and total protein analysis since the two receptor subtypes are definitely coexpressed in both the uterus and ovary [Kuiper et al., 1997; Couse et al., 1997; Brandenberger et al., 1997]. In contrast, we were unable to detect the clear predominance of ER β protein concentration that would be anticipated in ovary by transcript distribution

analysis [Kuiper et al., 1997]. Uterine ER β mRNA levels from different species have been reported to be much lower in comparison [Couse et al., 1997]. The essays herein were carried out using proteins extracted from whole tissue. Therefore, information is not available with respect to possible selective expression of the two ER forms within distinct cellular types. Immunohystochemical detection of ER α and β in rat ovary and uterus revealed nuclear and cell-type specific labeling [Hiroi et al., 1999; Sar and Welsch, 1998; Fitzpatrick et al., 1998]. For instance, uterine ER β protein was expressed predominantly by the glandular epithelium [Saunders et al., 1997; Hiroi et al., 1999].

The association of steroid receptors with non-nuclear/-cytoplasmic components has been previously documented. In pancreatic acinar cells, estrogen binding proteins were immunocytochemically identified all over the endoplasmic reticulum surface, on the plasma membrane and inside mitochondria, while no labeling could be localized in the nucleus [Grossman et al., 1989]. Moats and Ramirez [1998] reported that after injection of a radioiodinated membrane-impermeant 17β-estradiol conjugate a great proportion of the initial radioactivity proved to be selectively associated within seconds to liver microsomal/plasmalemmal subcellular fractions. A subsequent rapid delivery of estrogen binding components from the plasmalemma into mitochondria, and not into the nucleus, was also demonstrated. No information was given on the suspected ER subtype involved in this process. In a more recent work, the same authors [Moats and Ramirez, 2000] showed that HepG2 cells could specifically bind a gold-labeled macromolecular 17β-estradiol derivative by clathrin-coated pits, with a subsequent rapid translocation of the label to intracellular organelles resembling vesiculated mitochondria.

The present observations may lend support to the known effects of various steroids on mitochondrial physiology and gene transcription regulation. Thus, evidences have been obtained about tamoxifen direct actions on mitochondrial membrane functions. Tamoxifen prevented in a dose dependent manner the "mitochondrial permeability transition" characterized by membrane depolarization, matrix calcium release and amplitude swelling of the organelle [Custodio et al., 1998]. Since the nature of these effects could not be attributed to the antioxidant properties of tamoxifen, the possible involvement of a receptor should not be excluded. These data parallel very closely with independent descriptions [Pasqualini et al., 1986] on tamoxifen inducing enlargement of uterine mitochondria. The participation of classical ERs mediating tamoxifen biological actions was suggested by the authors, since an antibody that recognized the activated form of the ER also labeled [^{3}H]tamoxifen complexes. In a recent report, Chen et al. [1999] described a dose- and time-dependent increase in respiratory chain activity after estradiol treatment. The effect was inhibited by the specific antiestrogen ICI182,780. Estradiol and estrogen metabolites were able to increase the levels of mitochondrial genome-encoded transcripts in human hepatoma HepG2 cells [Chen et al., 1998]. Estrogens could also affect mitochondrial calcium retention by inhibiting the Na-dependent Ca^{2+} efflux from rat brain mitochondria [Horvat et al., 2000]. A rapid translocation of glucocorticoid receptors (GR) from the cytoplasm into mitochondria after administration of glucocorticoids, was demonstrated by Western Blotting and electron microscopy techniques [Demonacos et al., 1993]. GRs were able to directly interact with putative mitochondrial response elements, giving additional support to the well documented effects of glucocorticoids on mitochondrial function [Demonacos et al., 1995]. Similar results were obtained for thyroid hormone receptor localization, import and binding to hormone response elements [Demonacos et al., 1995].

Using an artificial experimental model, Razandi et al. [1999] demonstrated that a single transcript was capable of producing both membrane and nuclear residing receptors. Coupling to carrier proteins or post-translational modifications of some ER proteins could account for targeting to membranes or organelles. The addition of lipid anchors by palmitoylation or myristoylation would likely promote movement to membranes. Indeed, when we performed a search of ER α and β known sequences (from the Gene Bank) and introduced them into the program MOTIF (available from Internet), no obvious potential palmitoylation sites were evidenced. However, 4 to 7 consensus myristoylation sequences were found to be conserved among different mammalian species (human, rat and mouse), i.e. GVWSCE and GMX_1KCG, where X_1= M for ER α or V for ER β.

The present investigations indicate that a substantial proportion of the native [^{3}H]17β-estradiol binding sites in rabbit uteri, corresponding to the α and β ER proteins, are differentially associated to microsomal and mitochondrial membranes. The employment of more sensitive techniques should address the precise intracellular localization and dynamics of the ER proteins. Furthermore, future studies should clarify whether these results could be extensive to other tissues or cellular systems.

Finally, it has been previously suggested that ER α would be the predominant receptor isoform in reproductive organs, though ER β has proven to be a more widely expressed receptor among different tissues [Gustafsson, 1999]. A dominant role of ER α in the uterus was expected, since most characterization and purification studies on estrogen binding proteins were generally carried out on uterine tissue sources. However, our results from Ligand Blotting, clearly show that ER β proteins, though differentially localized in comparison to ER α, account for a significant proportion of total uterine estrogen binding sites. Indeed, ER β-related estrogen binding proteins appeared to be highly expressed in both rabbit uterus and ovary. We find amazing to consider that demonstration of the existence of another ER isoform was delayed until recent years.

ACKNOWLEDGMENTS

We specially acknowledge Dr. Kirk Riemer (University of California, San Francisco, USA) for donating the Syrian Hamster uterine Myocyte cell line. We thank Vet. Guillermo Lemus for generously providing the uterine tissue source, and Dr. Vincent Giguere (Royal Victoria Hospital, Montreal, Quebec, Canada) for providing the pCMXmER β expression vector. We are grateful to Zeneca Pharmaceuticals for donating the compound ICI 182,780. Paula Monje is a recipient of a CONICET research fellowship.

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