Estrogen receptors (ERα/ERβ) in normal and pathological growth of the human myometrium: pregnancy and leiomyoma

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Human myometria

Smooth muscle cells exhibit remarkable plasticity during normal and pathological development, as their phenotype can change rapidly and reversibly in response to changes in their hormonal environment. Pregnancy and leiomyoma, a mononuclear benign myometrial tumor, both lead to major morphological and biochemical modifications in the myometrium, illustrating the difference between the normal and pathological development of this tissue. There is compelling evidence that steroid hormones, especially estrogens, have a central role in modulating the growth, differentiation, and functions of target tissues like the myometrium (10). These hormones are produced in large amounts by the placenta during pregnancy, and their pleiotropic actions are critical to optimal myometrial responses for a successful pregnancy outcome. Leiomyoma is another estrogen-sensitive form of myometrial growth. This disorder is rare before puberty but is the most frequent benign uterine smooth muscle cell tumor in women of reproductive age. Leiomyoma can also rapidly increase in size during pregnancy, leading to fetal wastage, whereas they regress after menopause, ovariec-tomy, or gonadotropin-releasing hormone agonist therapy (32).

Classically steroid hormones act via specific intracellular receptor proteins (7, 20). There is no real consensus about the myometrial steroid receptor status during human gestation. Some have reported the presence of estrogen and/or progesterone receptors in the myometria of pregnant women at the end of pregnancy while others have not found them (29, 18, 31). There are also conflicting data on the relative abundance of steroid receptors in leiomyoma, with reports of overproduction or no change compared with normal tissue (4, 24, 33). It is thus important to know how the estrogen-receptor (ER) status changes during the normal and physiopathological growth of the human myometrium. This is particularly significant since several studies have shown that both leiomyoma and myometrium of pregnant women, especially at the end of pregnancy, show similar overexpression of a number of genes regulated by steroid hormones, such as the genes coding for connexin-43, insulin-like growth factors, epidermal growth factors, c-myc, and collagen. This had led certain authors to consider leiomyoma as a “pseudopregnant” myometrium (2).

Steroid hormone receptors are nuclear receptors that continuously shuttle between the nucleus and the cytoplasm (16). They are hormone-activated transcription factors that regulate the expression of specific genes by binding to steroid-responsive elements. Until recently, it was generally accepted that there was only one ER gene coding for the classical ER (ERα), which binds estrogen with high affinity. The description of a new gene encoding a second type of ER, termed ERβ (13), has prompted reevaluation of the estrogen signaling system. This new full-length ERβ cDNA was cloned from the human ovary and testis (23, 27). The chromosomal locations of the ERα and ERβ genes indicate that there are two independent ER genes in humans (13). Both of these forms of ER have been found in steroid-sensitive tissues (13, 19, 22, 23, 27), showing the complexity of the tissue response to estrogen. This also increases the likelihood that any selective effect of estrogen could be due to the differential expression of these two ER genes, depending on the physiological and/or physiopathological state of the target tissues.

Knowledge of the distributions of ERα and ERβ in the human myometrium is needed for studies on the respective function and importance of these ER sub-

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types in normal and abnormal growth. We have therefore compared the pattern of ER (ER\(\alpha\) and ER\(\beta\)) expression in the myometria of pregnant and nonpregnant women and in leiomyoma. We first analyzed the distributions of the ER\(\alpha\) and ER\(\beta\) mRNAs using RT-PCR and then determined the concentrations and affinity constants of the ER in these three tissues by ligand binding. Our results indicate that both ER\(\alpha\) and ER\(\beta\) mRNAs are present in the myometria of nonpregnant and pregnant women at term and in leiomyoma, but the amounts of these mRNAs and the estrogen-binding properties change according to the physiological and physiopathological growth of the myometrium.

MATERIALS AND METHODS

Tissues. Biopsies of myometria from pregnant women were obtained from patients (\(n = 8\)) with normal pregnancy who were delivered by elective cesarean section. The cesarean section was done for previously diagnosed cephalopelvic disproportion before the onset of labor between the 38th and 40th wk of amenorrhea. This procedure was approved by the Consultative Committee of Persons Involved in Biomedical Research of Paris-Cochin, and all subjects gave their informed written consent.

Normal and pathological (leiomyoma) nonpregnant myometrial samples were obtained from cyclic women (\(n = 10\)) aged 39–51 yr, undergoing hysterectomy for benign gynecological indications. Tissue samples were excised from normal muscle areas free of macroscopic visible abnormalities (\(n = 10\)). Samples of intramural leiomyoma (\(n = 5\)) were collected as previously described (5), and the uterus was examined by a pathologist to exclude adenomyosis or malignant changes. All of the leiomyoma were similar in size (2-cm diameter). None of the patients had been on hormonal medication for at least 3 mo before hospitalization. Surgery was scheduled during the luteal or follicular phases of the menstrual cycle. The stage of the menstrual cycle was estimated histologically by dating the endometrium (5). The biopsies were immediately frozen in liquid nitrogen and were stored at −80°C.

RNA preparation and RT. All reagents used for RNA isolation were purchased from Sigma Chemical (St. Louis, MO). Total RNA was isolated from myometria of pregnant (\(n = 5\)) or nonpregnant (\(n = 5\)) women and leiomyoma (\(n = 5\)) using an acid guanidinium-phenol-chloroform procedure (9). RT was performed using random hexanucleotides (20 µM) as primers on 4 µg of total RNA plus 200 µl Maloney murine leukemia virus RT in a final volume of 25 µl at 39°C for 60 min, as in the manufacturer’s specifications (Life Technologies). The cDNA products were stored at −20°C until required for PCR.

PCR. The primers used for human ER\(\alpha\) cDNA were as follows: ER\(\alpha\) upper (sense) 5'-CAG GGG TGA AGT GGG GTC TGC TG-3' (priming site in exon 4, nucleotides 1060–1082 as numbered in Ref. 27) and ER\(\alpha\) lower (antisense) 5'-TTT CCT TTT ACT GTC TTC TG-3' (nucleotides 1402–1422) with 0.1 mM of each dNTP, 0.4 µM primers, 1 mM MgCl\(_2\), and 2.5 U Taq polymerase (Life Technologies) in 25 µl PCR buffer. The amplification profile was 30 cycles with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

An aliquot (15 µl) of each PCR product was separated by electrophoresis (3% NuSieve agarose gel in Tris-borate-EDTA buffer containing 0.01% ethidium bromide). A molecular weight standard (lambda 123-bp DNA ladder; Life Technologies) was used to confirm the predicted PCR product sizes. Lack of genomic DNA contamination was checked in all experiments by conducting a control reaction containing mRNA without RT. Normalization of mRNA amounts in all samples studied was checked by amplifying an endogenous gene, human β2-microglobulin cDNA, using an additional pair of primers (17). The PCR products were also checked by Southern blot analysis with specific internal oligonucleotides for each sequence, 5'-TAGAAGGTTGATCATAGGCAGGG-3' for ER\(\alpha\) cDNA and 5'-CAGGAGCATCAGGAGGT-3' for ER\(\beta\) cDNA. Hybridization was performed with the specific probes labeled with fluorescein-11-desoxy-UTP using an enhanced chemiluminescence 3’ oligolabeling and detection kit (Amersham) according to the manufacturer’s instructions.

The intensities of the specific bands for ER\(\alpha\), ER\(\beta\), and β2-microglobulin mRNAs were analyzed densitometrically (Gel Scan DU Series 600 spectrophotometer; Beckman Instruments). Results were expressed as relative levels of specific mRNAs normalized to β2-microglobulin mRNA in each amplified sample.

Preparation of protein fractions. Each frozen sample of myometrium was thawed on ice and homogenized in ice-cold 12 mM Tris, 1.5 mM EDTA, 10% glycerol, and 2 mM Na\(_2\)Mo\(_4\) (pH 7.4) using a Polytron homogenizer and a tissue-to-buffer ratio of 1:10 (wt/vol). The homogenate was centrifuged at 1,000 g for 10 min, and the supernatant was transferred to an ultracentrifuge tube and centrifuged at 105,000 g for 60 min. The resulting cytosol was used in receptor-binding assays, as this procedure extracted a large percentage of the nuclear untransformed receptors (14, 22). All cytosol fractions were kept at −80°C. The protein concentration in each cytosol was determined by the dye-binding assay (Bio-Rad, Richmond, CA) with BSA as the standard.

Binding analysis of ER. Before the binding assay, the cytosol samples were treated with a dextran-coated charcoal pellet (10% NoritA and 0.1% dextran T70) for 1 h at 4°C to remove endogenous steroids. Aliquots of charcoal-treated cytosol (500 µg proteins) were then incubated (16 h at 4°C) with increasing concentrations (0.25–12 nM) of highly tritiated 17β[2,4,6,7-3H]estradiol (85–110 Ci/mmol; Amersham) or labeled estradiol plus a 500-fold molar excess of unlabeled diethylstilbestrol. The final volume of the incubation mixture was 0.5 ml. A 10-µl aliquot was then taken to measure total radioactivity. Bound and free hormone fractions were separated by adding an equal volume of a suspension of 1% charcoal and 0.1% dextran (wt/vol) for 10 min at 0°C and centrifugation at 1,500 g for 10 min. Aliquots of supernatant were counted in 4 ml of Ultima-gold XR scintillation fluid (Packard) in a Beckman LS 6000-I scintillation counter.

The binding parameters, affinity constant (\(K_d = 10^M\) M), and binding capacity (equivalent to total number of binding sites: fmoI/mg protein) were estimated by Scatchard graphical analysis after subtraction of the nonspecific binding. When more than one type of binding site was detected (biphasic curves), the Rosenthal graphical correction was used.
applied to determine the binding parameters of the first class of binding sites.

Statistical analysis. The nonparametric Mann-Whitney test for unpaired samples was used to compare the levels of ERα and ERβ mRNAs in the myometria of pregnant and nonpregnant women. The Wilcoxon signed-rank test for paired samples was used to determine significant difference in the levels of ERα and ERβ mRNAs between the leiomyoma and nonpregnant myometria.

The Mann-Whitney test for unpaired samples was also used to compare the kinetic parameters in the myometria of pregnant and nonpregnant women and in leiomyoma. All results are expressed as means ± SE.

The Statview statistical package for Mackintosh (Abacus Concepts, Berkeley, CA) was used in these analyses. P values of <0.05 were considered significant.

RESULTS

ERα and ERβ mRNAs in the myometria of pregnant and nonpregnant women and in leiomyoma. To determine the distribution of ERα and ERβ mRNAs in the myometria of pregnant and nonpregnant women and in leiomyoma, total RNAs from each tissue were reverse transcribed. The resulting cDNAs were amplified by PCR using a set of primers specific for each ER cDNA sequence. As illustrated in Fig. 1, electrophoresis on agarose gel revealed PCR products of the predicted sizes 483 bp for ERα and 259 bp for ERβ in all tissues. The successful normalization of RNA amounts during the RT step was verified by amplification of a fragment of the reference standard, β2-microglobulin cDNA, in all of the samples analyzed (Fig. 1).

The apparent amounts of ERα and ERβ mRNAs in the three tissues were compared after Southern blotting and hybridization with oligonucleotide probes specific for each ER subtype (Fig. 2A). Densitometric analysis of the signals indicated wide variations in the ERα and ERβ (Fig. 2B) mRNA levels in normal myometrial samples from different nonpregnant patients without any evident correlation with the stage of the menstrual cycle. A comparison of the signals in the myometrium and the leiomyoma from the same patient indicated that these signals were increased (3- to 8-fold) for ERα mRNA in three leiomyoma (Fig. 2B, lanes 2–3, 11–12, and 14–15), whereas the signals for the two other patients were more similar (Fig. 2B, lanes 5–6 and 8–9; P < 0.06, leiomyoma vs. nonpregnant myometria). The weakest signals for ERα mRNA were from the pregnant myometria (P < 0.01, pregnant vs. nonpregnant groups).

The signals for ERβ transcripts also appeared to be greater (1.5- to 4-fold) in leiomyoma (Fig. 2B, lanes 2–3, 5–6, 8–9, and 14–15) than in nonpregnant myometria, except for one case (Fig. 2B, lanes 11–12; P < 0.06, leiomyoma vs. nonpregnant myometria). Homogenous amounts of ERβ mRNA were found in pregnant myome-
tria except for in one woman (Fig. 2B, lane 1). Globally, the intensities for ERβ mRNA in pregnant and nonpregnant myometria were similar.

Binding properties of ER in the myometria of pregnant and nonpregnant women and in leiomyoma. The binding patterns of untransformed ER isolated from myometrial cytosol of pregnant or nonpregnant women and from leiomyoma are shown in Fig. 3. Table 1 summarizes the binding kinetic values with the $K_a$ and number of binding sites ($n$) determined after graphical Scatchard analysis. The specific binding of 17$\beta$-estradiol to the myometrial receptors in nonpregnant women followed a biphasic saturation curve (Fig. 3) with a curvilinear Scatchard plot, indicating two binding components: one class of binding sites (I) with high affinity [$K_a(I) = 4.8 \pm 0.6 \times 10^8$ M$^{-1}$] and low capacity [$n(I) = 10.2 \pm 1.6$ fmol/mg protein] and a second class (II) with a lower affinity [$K_a(II) = 0.3 \pm 0.1 \times 10^9$ M$^{-1}$] and a greater capacity [$n(II) = 122 \pm 15$ fmol/mg protein]. The stage of the menstrual cycle had no evident influence on these binding parameters under our experimental conditions. By contrast, linear transformation of saturation data revealed a single population of 17$\beta$-estradiol-binding sites in the myometria of pregnant women. High-affinity binding sites, typical of type I, were not detected in pregnant myometria (Fig. 3). Only the low-affinity binding sites ($K_a = 0.2 \pm 0.1 \times 10^9$ M$^{-1}$) with high capacity ($n = 114 \pm 13$ fmol/mg protein) were found. Similarly, a single population of low-affinity binding sites ($K_a = 0.3 \pm 0.1 \times 10^9$ M$^{-1}$) was found in leiomyoma (Fig. 3). Their concentration was two- to threefold greater ($n = 273 \pm 38$ fmol/mg protein) than the concentration in the nonpregnant (P < 0.001) or pregnant (P < 0.003) myometria.

**DISCUSSION**

This study describes the specific phenotypes of human myometria in pregnancy and leiomyoma, in terms of ER subtypes. The distributions of ERα and ERβ mRNAs and the binding properties of the resulting ER in late-pregnancy myometria and in leiomyoma reveal certain similarities and differences between the normal and pathological growth of the human myometrium. We first analyzed the ER status according to the physiological development of the human myometrium in pregnancy. mRNAs for both the classical ERα and the new ERβ were detected in the myometria of pregnant and nonpregnant women, but their patterns differed with the tissue studied. The RT-PCR analysis clearly indicated that the myometria of pregnant women...
contains little mRNA encoding ERα, whereas the amounts of ERβ mRNA in pregnant and nonpregnant tissues appear to be similar. The binding characteristics of the ER emphasize the changes occurring in the myometrium from pregnant women. Thus the nonpregnant myometria contains two specific 17β-estradiol-binding sites, one with a high affinity and low capacity and a second with a lower affinity and greater capacity. In contrast, the myometria of pregnant women appear to contain no high-affinity binding sites and only the lower-affinity sites at the same concentration as in nonpregnant myometria. These data are compatible with previous results obtained using sucrose density gradient centrifugation and titration analyses (18, 29, 31).

Our second interest is in a physiopathological development of the human myometrium, the leiomyoma, which is the most common uterine tumor and a major public health problem since it is often an indication for hysterectomy. The increases in ERα mRNA and ER protein in leiomyoma are controversial (4, 24). In agreement with previous studies (4) and although there are wide variations in ER mRNA levels in myometrial samples from different patients, our results indicate that the amounts of ERα mRNA may well be enhanced in leiomyoma. There also appears to be more ERβ mRNA in leiomyoma. These observations might be explained by the fact that the region 14q-22—24 of chromosome 14, which includes the ERβ gene, is involved in rearrangements in uterine leiomyoma (13). The ER-binding studies revealed only low-affinity estrogen-binding sites in leiomyoma. The number of these low-affinity binding sites is two- to threefold greater in leiomyoma than in normal nonpregnant myometria (P < 0.001). Previous reports also indicate an increase in estrogen-binding sites in leiomyoma (4). The increase in the number of ER in leiomyoma may account for their enhanced sensitivity to estrogen (26).

Recent studies indicate that the ERβ subtype differs from the ERα subtype in the COOH-terminal ligand binding (58% amino acid sequence homology) and the NH₂-terminal transactivation domains, although the two ER subtypes have nearly identical DNA binding domains (96% homology; see Refs. 13, 19, 22, 23, 27). However, the ERα and ERβ subtypes can give opposing regulatory signals to 17β-estradiol from the same DNA response element at activator protein 1 sites. Thus 17β-estradiol bound to ERα activates transcription, whereas this natural hormone bound to ERβ inhibits transcription (30). There are also differences in the binding affinities of the ER subtypes for estrogenic substances. ERβ receptors have a lower affinity for 17β-estradiol than do ERα receptors (22). Our findings show not only parallels between the little mRNA encoding ERα and the lack of high-affinity binding sites in the myometria of pregnant women but also between the presence of ERβ mRNA and the persistence of low-affinity estrogen-binding sites in this tissue. It is thus

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Table 1. Binding parameters of estrogen receptors in the myometria of pregnant and nonpregnant women and in leiomyoma

<table>
<thead>
<tr>
<th>Estrogen Receptors</th>
<th>Pregnant Myometria</th>
<th>Nonpregnant Myometria</th>
<th>Leiomyoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kᵦ, (10^9) M⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4.8 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>II</td>
<td>0.2 ± 0.1</td>
<td>10.2 ± 1.6</td>
<td>273 ± 38†</td>
</tr>
<tr>
<td>n, fmol/mg protein</td>
<td>114 ± 13</td>
<td>122 ± 15</td>
<td></td>
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All results are expressed as means ± SE. Binding kinetic values were determined from Scatchard graphical analysis of Fig. 3. Apparent affinity constants (Kᵦ) and binding capacities (n) for myometria of pregnant (n = 8) and nonpregnant women (n = 10) and for leiomyoma (n = 4) were compared using the nonparametric Mann-Whitney test for unpaired samples. I, high-affinity, low-capacity class of binding sites; II, lower affinity, greater capacity class of binding sites. *Leiomyoma vs. nonpregnant myometria, P < 0.001; †leiomyoma vs. pregnant myometria, P < 0.003.
possible that the ERα corresponds to the classical 17β-estradiol high-affinity binding sites (15) and that ERβ is equivalent to the low-affinity binding sites. This notion is also consistent with the apparent elevated ERβ mRNA and the increased number of low-affinity estradiol-binding sites in leiomyoma compared with nonpregnant myometrium. Nevertheless, the discrepancy between the presence of substantial ERα mRNA in leiomyoma and the apparent lack of high-affinity estradiol-binding sites in this tumor must be clarified. This might result from inappropriate relative proportions of the two ER subtypes in leiomyoma. The range of ligand concentrations used in this study was wide and normally sufficient to distinguish between a mixed population of receptor subtypes with sufficiently different affinities (10- to 100-fold), but it is possible that the overabundance of low-affinity sites in leiomyoma may mask the presence of high-affinity binding sites. In addition, there could be ERα variants lacking part of the hormone-binding domain in leiomyoma, as shown recently in breast cancer (28). Last, posttranscriptional modification or faulty translation of the ERα mRNA into functional protein is always possible.

Hence, what is the functional significance of the presence of ERβ mRNA and the low-affinity ER subtype in pregnant myometria and leiomyoma? The role of the ERβ may well differ depending on the presence or absence of the classical ERα. Recent studies indicate that ERα/ERβ heterodimers are apparently formed in the preference to homodimers (11). This opens the possibility that ERα and ERβ act synergistically or as antagonists via (homo or hetero) dimerization and activation of a common responsive element. The relative importance of the ERα and ERβ subtypes in uterine development and function is shown by studies using ERα knockout (ERKO; see Refs. 11 and 21) mice. The ERKO mice, in which the amount of ERβ is not significantly altered, survive but are infertile. ERKO mice also have abnormally high baseline plasma levels of gonadal hormones (10-fold higher 17β-estradiol than in wild mice; see Ref. 11). The weak ERα mRNA signal and the presence of ERβ mRNA, plus the high circulating estrogen concentration in women in term pregnancy, appear to be remarkably similar to the endocrine situation in ERKO mice.

Another question is how does estrogen still exert its physiological action, despite the apparent lack of high-affinity binding sites? There is still no clear consensus as to how steroid receptors act or the precise function of the low-affinity binding sites. These latter sites have been found in normal and malignant mammalian tissues (36) and have been proposed to be involved in estrogen-induced cell growth (25). It has also been suggested that they bind not only estrogen but other ligands and inhibitors of cell proliferation as well (25). Recent reports indicate that ERβ preferentially binds environmental phytoestrogens like genistein (22), and several studies indicate that genistein may regulate cell growth (1, 3, 35) and mainly binds to these sites in uterine and mammary tumor cells (3). The effect of such environmental estrogens on the reproductive ability of mammals is also debated, and there has been speculation that some of their claimed effects on fertility are mediated via ERβ (13). It is, perhaps, not so surprising that low-affinity binding sites and ERβ mRNA are substantially found during pregnancy and in leiomyoma, since both situations lead to a relative infertility and involve myometrial hypertrophy and/or hyperplasia. The growth of the myometrium during periods of increased estrogen production, such as pregnancy, is thought to be primarily due to cell hypertrophy, resulting in an increase in cell volume (6, 8). Thus the hypertrophy of the smooth muscle cells that occurs mainly during pregnancy is accompanied by an increase in contractile proteins and reorganization of intracellular organelles (8). On the other hand, the increased myometrial growth in response to estrogen in leiomyoma is mainly due to proliferation of smooth muscle cells and could reflect altered estrogen responsiveness (6).

The influences of ERα and ERβ and their relative proportions in sustaining myometrial hypertrophy and proliferation, together with the actions of other effec tors, such as progesterone and growth factors, require further clarification. However, the changes in the ERα and ERβ balance during pregnancy and in leiomyoma described in this study shed some light on the long-standing puzzle of the uterotropic response to steroid hormones mediated via specific steroid-regulated genes.

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