The estrogen receptor α $\Sigma 3$ mRNA splicing variant is differentially regulated by estrogen and progesterone in the rat uterus

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Abstract

The gene for estrogen receptor α (ER α) has been shown to be under complex hormonal control and its activity can be regulated by mRNA alternative splicing. Here we examined the regulation of ER α transcription and translation in the rat uterus by ovarian steroid hormones. We examined whether expression of ER α mRNA splice isoforms is hormonally regulated in ovariectomized (OVX) and cycling rats. Adult OVX female rats were treated daily with 17- β estradiol (E₂) (0.05 μ g/rat or 5 μ g/rat), progesterone (P₄) (1 mg/rat) or a combination of both hormones for 4 days. Animals were killed 24 h after the last injection and uterine horns were removed. In order to determine whether ER α mRNA isoforms are differentially expressed under various physiological conditions, animals were evaluated at proestrus, estrus and diestrus. The ER α protein and mRNA were detected by immunohistochemistry and comparative RT-PCR analysis respectively. The presence of ER α mRNA isoforms was evaluated using a nested RT-PCR assay. In OVX control rats, ER α mRNA and protein levels were high, demonstrating a constitutive expression of the ER α gene in the uterus. When animals received P₄ or the high dose of E₂,

a significant decrease in both ER α mRNA and protein was observed in the uterus. However, when rats were treated with the low dose of E₂, only the ER α protein was down-regulated; no changes were observed in ER α mRNA expression. In addition to the full-length ER α mRNA, OVX control rat uteri expressed three shorter transcripts: $\Sigma 3$, $\Sigma 4$ and $\Sigma 3,4$ (lacking exon 3, exon 4, or both 3 and 4 respectively). Surprisingly, when OVX animals were treated with P₄, the low dose of E₂ or a combination of both steroids, expression of the $\Sigma 3$ isoform was completely abolished. During the estrous cycle, all ER α mRNA splicing variants were detected at proestrus and estrus. However, in diestrus, significant low levels of the $\Sigma 3$ isoform were observed. In summary, our results suggest a dose-dependent relationship between E₂ concentrations and the level of control in the ER α transcription-translation cascade. Moreover, the alternative splicing of the ER α primary transcript is influenced by the hormonal milieu, suggesting that these events could affect the estrogen responsiveness of the rat uterus during the estrous cycle.

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Introduction

Two different genes coding for the estrogen receptor (ER) have been identified: the classical ER α (Koike *et al.* 1987) and the more recently characterized ER β (Kuiper *et al.* 1996). 17- β Estradiol (E₂) binds ER α with a higher affinity than ER β , therefore promotes higher rates of ER α -mediated transcriptional activity at the estrogen response element (ERE) (Pettersson *et al.* 2000). Moreover, ER α and ER β may have entirely opposite transcriptional effects at activating protein-1 (AP-1) sites, depending upon the structural properties of the ligand (Paech *et al.* 1997).

The rat uterus, a major target tissue for ovarian steroids, has served as an excellent model for studying hormonal

regulation of ER α expression. Both, up-regulation and down-regulation of ER α by E₂ has been reported in the rat uterus, depending upon the physiological state of the animal and/or the experimental system employed (Zhou *et al.* 1993, Wang *et al.* 1999, Nephew *et al.* 2000). Since receptor levels influence target tissue responsiveness to the hormonal milieu, there has been a great interest in the understanding of how the ER is regulated.

It has been shown in the rat uterus that progesterone (P₄) selectively decreases ligand-bound ER α and inhibits recovery of ER α at transcriptional and translational levels (Clarke & Sutherland 1990). The repression of ER α -mediated transcriptional activity and the inhibition

of ER α retention by P₄ are mediated by the ligand-bound progesterone receptor (PR) (Kraus *et al.* 1995).

A great molecular diversity of ERs is achieved by the alternative splicing of mRNA isoforms that have been described in a variety of cells and tissues, particularly in cancer cell lines and tumors (Hopp & Fuqua 1998). Several of the variant transcripts generated by an exon skipping mechanism of the primary ER α pre-mRNA retain the same reading frame as the full-length transcript, and the corresponding variant proteins have been detected *in vivo* and *in vitro* (Pfeffer *et al.* 1995, Poola *et al.* 2000). It has been shown that the ER α splice variants lacking exon 3 and/or 4 (defined as Σ 3, Σ 4 and Σ 3,4) are all translated into proteins *in vivo*; moreover, their expression levels varied during rat pituitary ontogeny and also according to the sex of the animal (Pasqualini *et al.* 1999). In addition, these splice variants were also observed in the rat uterus as exon-deleted transcripts (Friend *et al.* 1997). There is little information in the literature regarding steroid hormonal regulation of ER α mRNA isoform expression. Moreover, it has been suggested that ER mRNA splice variants might contribute to the deregulation of growth and differentiation in premalignant and malignant tissues in endocrine-related tumors (Hopp & Fuqua 1998).

In the present study, we examined the effects of E₂ and P₄ on the control of ER α transcription and translation in the uterus of adult ovariectomized (OVX) rats. The same animal model was used to determine whether these steroid hormones regulate the expression pattern of ER α mRNA splice variants. Furthermore, we studied if these isoforms are differentially expressed during the rat estrous cycle.

Materials and Methods

Experimental design

Sexually mature female rats of an inbred Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 $\pm$ 2 °C; lights on from 0600 to 2000 h) and had free access to pellet laboratory chow (Constantino, Córdoba, Argentina) and tap water. All rats were handled in accordance with the principles and procedures outlined in the FRAME's guidelines (<http://www.frame.org.uk/reductioncommittee/journalguidelines.htm>).

For steroid treatments, female rats were OVX and then rested for 14 days. Rats that exhibited at least 7 days of atrophic vaginal smears (Montes & Luque 1988) were injected s.c. daily for 4 days with vehicle (sesame oil), E₂ (0.05 or 5 μ g/rat), P₄ (1 mg/rat) or a combination of both steroids. Rats were included in one of six experimental groups (n =7–8 rats/group): sesame oil (control), low dose of E₂ (0.05 E), high dose of E₂ (5 E), P₄ (P) or combinations 0.05 E+P and 5 E+P. The low dose of E₂ and the dose of P₄ were chosen because they reproduced

the physiological plasma levels described in pregnant rats (Downing & Sherwood 1986, Luque *et al.* 1996). Moreover, a 100 times higher dose of E₂ was also tested. Animals were killed 24 h after the last injection and uteri were isolated. One uterine horn from each rat was placed immediately in liquid nitrogen and stored at –80 °C for RNA extraction. The other uterine horn was fixed by immersion in 10% buffer formalin for 6 h at 4 °C, embedded in paraffin, and used for immunohistochemical staining.

Intact 12-week-old female rats were used to determine expression of uterine ER α splice isoforms during the normal estrous cycle. Daily vaginal cytology was examined to establish different stages of the estrous cycle (Montes & Luque 1988). Only rats exhibiting three consecutive regular estrous cycles were employed. Animals were killed and uteri collected between 1000 and 1100 h at proestrus, estrus or diestrus (n =5 rats per stage of estrous cycle). The uterine horns from each rat were placed immediately in liquid nitrogen and stored at –80 °C for RNA extraction.

ER α expression detected by immunohistochemistry

Serial sections (5 μ m in thickness) of paraffin-embedded uterine horns were mounted on 3-aminopropyl triethoxysilane (Sigma)-coated slides and dried for 24 h at 37 °C.

A standard immunohistochemical technique (avidin–biotin–peroxidase) was used to visualize ER α immunostaining intensity and distribution. In brief, for detection of ER α , a microwave (MW) pretreatment for antigen retrieval was performed following the protocol previously described (Muñoz-de-Toro *et al.* 1998). After washing in buffer (0.05 M PBS, pH 7.5), endogenous peroxidase activity and non-specific binding sites were blocked. Sections were incubated overnight at 4 °C in a humidified chamber with a diluted mouse monoclonal antibody raised to the full-length recombinant human ER α (working dilution 1:60; clone 6F11, Novocastra, Newcastle upon Tyne, UK), followed by incubation with a biotinylated secondary antibody (Sigma). Negative controls were obtained by replacing the primary antibody with non-immune mouse serum (Sigma). The reaction was developed using the avidin–biotin–peroxidase method and diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (PMMyR, Buenos Aires, Argentina).

Quantitation of ER α expression by image analysis

Image analysis was performed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as previously described (Ramos *et al.* 2002). In brief, images were recorded by a Sony ExwaveHAD color video camera attached to an Olympus BH2 microscope (illumination: 12-V halogen lamp, 100 W, equipped with a stabilized light source), using a Dplan $\times$ 100 objective

Table 1 Positions of primers and PCR product sizes for RT-PCR experiments

	Position of primers on cDNA	PCR product size (bp)
cDNA (GenBank reference ER α (NM_012689))	E1: 681–701 E2: 1003–1025	345
GADPH (AF106860)	GADPH1: 94–111 GADPH2: 439–462	466
ER α First round	E3: 819–841 E4: 1422–1442	Full length: 624, Σ 3: 507, Σ 4: 288, and Σ 3,4: 171
ER α Second round	E5: 836–856 E6: 1372–1389	Full length: 552, Σ 3: 435, Σ 4: 216, and Σ 3,4: 99

(numerical aperture, 1.25). The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and calibration of the measurement system were done with a reference slide. Using Auto-Pro macro language, an automated standard sequence operation was created to measure optical density (OD). In this automated analysis process, the images from immunostained slides were converted to a gray scale and the OD was measured as an average gray, being equal to the sum of the gray intensity of each pixel divided by the number of pixels measured. The resolution of the images was set to 640 $\times$ 480 pixels and the final screen resolution was 0.103 μ m/pixel.

For each uterine horn specimen, 3 sections separated at 20 μ m intervals were evaluated and 20 microscopic fields were analyzed in each tissue compartment (luminal epithelium, glandular epithelium, subepithelial stroma and muscular stroma).

ER α mRNA analysis by comparative RT-PCR

Reverse transcription and PCR amplification were performed following the technique previously described by Ramos *et al.* (2003) with minor modifications. Total RNA was isolated from frozen uterine tissue using Trizol reagent (Life Technologies). The total RNA concentration was assessed by A₂₆₀ and isolated RNA was stored at –80 °C until needed. Equal quantities (4 μ g) of total RNA from each animal were reverse-transcribed into cDNA with avian myoblastosis virus (AMV) reverse transcriptase (12.5 U; Promega) using 200 pmol of random hexamers (Promega) to prime the reaction; 20 U ribonuclease inhibitor (RNase OUT; Invitrogen) and 200 nmol of a deoxy NTP mixture were added to each reaction tube in a final volume of 30 μ l of 1 $\times$ AMV-RT buffer. Reverse transcription was performed at 42 °C for 90 min. Reactions were terminated by heating at 97 °C for 5 min. Reactions were next cooled on ice, followed by dilution of the reverse-transcribed cDNA with RNase-free water to a final volume of 60 μ l. RNA incubated under identical conditions, but without reverse transcriptase served as a negative control.

Oligonucleotides primers for PCR were synthesized by Invitrogen. The locations of primers on the cDNAs, the GenBank accession numbers and the size of amplified fragments are listed in Table 1. A pair of primers was used to quantify the ER α gene transcription level: sense primer, E1, 5'-AATTCTGACAATCGACGCCAG-3'; antisense primer, E2, 5'-GTGCTTCAACATTCTCCC TCCTC-3'. In addition, as an internal control of reverse transcription and reaction efficiency, amplification of GAPDH mRNA was carried out in parallel in each sample using the following primers: sense primer, GAPDH1, 5'-CAGCCGCATCTTCTTG-3'; antisense primer, GAPDH2, 5'-AGTTGTCATATTTCTC GTGGTTCA-3'. All amplifications were performed in duplicate. To perform comparative PCR, aliquots of cDNA samples equivalent to 800 ng total RNA input were used in each PCR amplification. Each reaction mixture contained: 2.5 U Taq-DNA polymerase (Promega), 1.5 mM MgCl₂ (Promega), 0.2 mM of the dNTPs mix (Promega) and 20 pmol of each primer in a final volume of 25 μ l of 1 $\times$ PCR Taq buffer. After initial denaturation at 97 °C for 2 min, the reaction mixture was subjected to successive cycles of denaturation at 96 °C for 1 min, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min. A final extension cycle at 72 °C for 10 min was included. The optimal number of cycles for each reaction was determined experimentally to yield linear relationships between signal intensity and cycle number (see Results).

ER α mRNA splicing variants analysis by RT-nested PCR

In order to amplify full-length ER α mRNA and the exon-deleted splicing variants Σ 3, Σ 4 and Σ 3,4 (lacking exon 3, exon 4, or both 3 and 4 respectively), we performed a nested-PCR with ER α -specific primers located upstream of exon 3 and downstream of exon 4 (Koike *et al.* 1987). We used nested-PCR to improve the specificity and accuracy of the PCR amplification process by making a second round of amplification using primers placed internally of the first pairs of primers (see Table 1). Reverse transcription of total RNA and PCR reaction

Table 2 OD of ERα-immunostained cells in luminal epithelium (LE), glandular epithelium (GE), subepithelial stroma (SS) and muscular stroma (MS)

Groups	ERα (OD)			
	LE	GE	SS	MS
C	49.7 ± 8.06	73.4 ± 7.78	42.2 ± 3.67	43.6 ± 7.31
5 E	18.5 ± 4.40**	12.5 ± 1.34**	25.4 ± 4.42**	37.7 ± 7.23
0.05 E	37.0 ± 3.21*	45.3 ± 10.3*	33.6 ± 2.13*	36.9 ± 6.96
5 E+P	56.9 ± 7.24	79.1 ± 11.9	41.0 ± 3.61	36.1 ± 7.41
0.05 E+P	45.7 ± 12.7	64.6 ± 12.6	46.5 ± 11.2	29.5 ± 6.94
P	10.3 ± 5.89**	72.5 ± 8.45	40.7 ± 8.13	21.6 ± 2.67*

Values are means ± S.E.M. Asterisks indicate statistically significant differences between control and hormone-treated groups (**P*<0.05; ***P*<0.01). C; control group.

mixtures was performed as described above. To perform the first round of amplification, the following pair of primers was used: sense primer, E3, 5′-GTCTGGTCCT GTGAAGGCTGCAA- 3′; antisense primer, E4, 5′-AGG AGCAAACAGGAGCTTCCC- 3′. The PCR conditions included an initial denaturation step at 95 °C for 30 s, followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. At the end of the cycling program, a final extension cycle at 72 °C for 5 min was included. Products from the first reaction were diluted 1/2000 and used in the second round of amplification with the following primers: sense primer, E5, 5′-CTGCAAGGCTTTCTTTAA GAG-3′; antisense primer, E6, 5′-TCATCAGGATCTC CAACC- 3′. The second round of PCR was performed using the same conditions as described for the first round.

Detection and identification of PCR products

The generated cDNA fragments were resolved in 1.5% agarose gels containing ethidium bromide (Sigma) and their molecular sizes determined by comparison with DNA standards (Cien Marker; Biodynamics, Buenos Aires, Argentina). Agarose gel images were digitized using a Sony ExwaveHAM color video camera (Sony Electronics Inc, Sony Drive, Park Ridge, NJ, USA) and the Image Pro-Plus 4.1.0.1 image system analyzer (Media Cybernetics, Silver Spring, MD, USA). GAPDH mRNA was selected as an internal control since expression of GAPDH mRNA remained constant in all experimental conditions. In comparative PCR analysis, the absolute OD values for each PCR product were obtained by densitometry and were normalized with GAPDH levels. Relative levels of the specific mRNAs were expressed in arbitrary units. In all assays, negative controls using RNA without reverse transcription and Taq-DNA polymerase-negative tubes were performed in order to minimize the introduction of potential artifacts. All PCR products were cloned

using the TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown).

Data analysis

The statistical analysis was performed by the Kruskal–Wallis one-way ANOVA and significance between groups was determined by Dunn’s post-test (Siegel 1956).

Results

Cell type-expression of ERα protein in the uterus of OVX rats

The relative ERα expression level in each uterine cellular compartment was determined by measuring the OD of nuclei in ERα-immunostained tissue sections (Table 2). OD levels for ERα in the uterus of control adult OVX rats were high, demonstrating a constitutive expression of ERα in epithelial, stromal and myometrial compartments (Fig. 1A). In controls, glandular epithelium showed the strongest signal, with all cell nuclei highly immunostained (Figs 1A and 2).

Treatment with E₂ resulted in significant changes in ERα expression patterns (Figs 1B and 2). OVX rats treated with E₂ exhibited a dramatic decrease in the intensity of ERα staining in the luminal epithelium, glandular epithelium and subepithelial stroma; in contrast, the high ERα level was maintained in the muscular compartment (Fig. 2). The E₂-induced down-regulation of ERα was detected with both low and high doses of E₂; however, a greater reduction in ERα was observed in the high dose-group (Fig. 2). Addition of P₄ to the E₂ treatment inhibited the down-regulation of ERα observed in E₂-treated animals (Fig. 1C). When OVX animals received P₄ alone, the immunohistochemical results revealed a clear down-regulation of ERα only in luminal epithelium and

Figure 1 Representative photomicrographs of immunohistochemical detection of uterine ER α from adult OVX rats injected with: (A) sesame oil (control); (B) E₂ (5 µg/rat); (C) E₂ (5 µg/rat)+P₄ (1 mg/rat); (D) P₄ (1 mg/rat). (A) The high constitutive expression of ER α in luminal and glandular epithelium, and subepithelial stroma from control rats is shown. (B) A significant reduction in ER α staining in luminal and glandular epithelium and subepithelial stroma in rats treated with the high dose of E₂ was observed. (C) Down-regulation of ER α observed in E₂-treated rats was lost when P₄ was added. (D) When animals received P₄ alone, immunostaining revealed a clear down-regulation of ER α in luminal epithelium and muscular stroma. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma. Scale bar represents 50 µm.

muscular stroma. No significant changes in glandular epithelium and subepithelial stroma were observed following P₄ administration (Figs 1D and 2).

Effects of E₂ and P₄ on total ER α mRNA in OVX rat uterus

Expression levels of ER α and GAPDH mRNAs in the uterus of experimental groups were evaluated by RT-PCR. Optimization of assays for semiquantitative analysis was carried out for each target by correlating the number of PCR cycles with the OD of the PCR products, using total RNA from a pool of control and treated rat uteri. This method revealed a linear relationship for amplification between cycles 28–36 for ER α and cycles 22–32 for GAPDH. All linear correlation coefficients were greater than 0.97. Based on these results, and in accordance with our previously published protocols (Ramos *et al.* 2003, Kass *et al.* 2004), we have chosen 30 and 28 cycles for

ER α and GAPDH respectively, using separate reactions for each target gene.

Representative ethidium bromide-stained images of ER α and GAPDH mRNA by RT-PCR analysis are shown in Fig. 3. The relative expression of ER α mRNA was determined in each uterus ($n=7-8$ animals/group), with the results showing a differential modulation dependent upon the hormonal treatment. An abundant ER α PCR product of the expected size (345 bp) was generated after 30 cycles from reverse-transcribed uterine mRNA from adult OVX rats (Fig. 3). The identity of this product was confirmed by cloning and sequencing the PCR products (data not shown). The high dose of E₂ produced a significant reduction in the level of ER α mRNA. In contrast, the expression of ER α mRNA was unaffected when animals received the low dose of E₂. In addition, there were no significant changes in OD of ER α mRNA when combining low or high doses of E₂ with P₄ compared with controls. However, when animals were

Figure 2 Quantitative measurement of ER α protein expression in different uterine cellular compartments following ovarian steroid treatment. Adult OVX animals were injected with sesame oil (C group), high dose of E $_2$ (5 E group), low dose of E $_2$ (0.05 E group), a combination of E $_2$ +P $_4$ (5 E+P group and 0.05 E+P group), or P $_4$ alone (P group). Bars represent means+s.e.m. ($n=7-8$ animals/group). Asterisks indicate significant differences between control and hormone-treated rats (* $P<0.05$; ** $P<0.01$).

injected with P $_4$ alone, a significant down-regulation of ER α mRNA expression was observed in comparison to controls.

The expression of alternatively spliced receptor variants in OVX rats following steroid treatment suggests a differential hormonal control

To investigate the presence of alternatively spliced receptor variants, we applied an RT-nested PCR procedure employing two pairs of specific oligonucleotides. The second-round PCR products revealed the presence of the full-length ER α mRNA (552 bp) in all samples. In addition, three exon-skipping isoforms could be detected with this protocol, the Σ 3 (lacking exon 3), Σ 4 (lacking exon 4) and the Σ 3,4 variant (lacking exons 3 and 4). The amplification products were subsequently cloned and sequenced and were shown to represent splicing variants of the entire ER α mRNA which could maintain the in-phase reading frame of the ER α .

In the uterus of control OVX animals, we detected the four transcripts with a predominant signal of the full-length amplification product (Fig. 4). When animals received the

high dose of E $_2$, a down-regulation of both the full-length ER α mRNA and also of the three exon-skipping isoforms was observed. However, when rats were treated with the high dose of E $_2$ plus P $_4$, no differences were observed in comparison to controls (Fig. 4). Surprisingly, in animals treated with either the low dose of E $_2$, P $_4$ alone or the combination of both hormones, the expression of the Σ 3 isoform was completely abolished (Fig. 4). Interestingly, nucleotide sequence analysis revealed that this isoform is an exon-skipping variant that lacks the coding region for the second Zn-finger of the DNA-binding domain.

Expression of ER α mRNA splicing variants depends upon the estrous cycle

As in the uterus of OVX control animals, the four ER α mRNA transcripts were detectable at all stages of the estrous cycle (Fig. 5). The pattern of expression was similar between proestrus and estrus, with a predominant expression of the full-length amplification product. Levels of the Σ 3 isoform were highest at proestrus and estrus, while in diestrus, a very low expression of this isoform was detected. The Σ 4 and Σ 3,4 isoforms did not exhibit

Figure 3 Expression of total ER α mRNA in the uterus of OVX control rats and following ovarian steroid treatment. Representative ethidium bromide-stained gel electrophoresis of uterine ER α mRNA from two independent samples per experimental group are presented. (A) Very low expression of ER α mRNA was exhibited by animals treated with the high dose of E₂ (5 E group) and with P₄ alone (P group), when compared with controls. (B) Parallel amplification of GADPH mRNA served as an internal control. In each gel, representative non-RT negative controls are presented (–). Abbreviations of experimental groups are detailed in legend of Fig. 2. (C) Densitometric values of ER α mRNA, expressed in arbitrary OD units relative to GADPH mRNA levels are shown. Bars represent mean values ($\pm$ S.E.M.) of at least 7 animals/group. Asterisks indicate significant ($P < 0.05$) differences compared with controls.

changes in the uterus with changes in the rat estrous cycle, suggesting that expression of these isoforms is not regulated by sex hormones.

Discussion

The mechanisms that control ER α expression remain to some extent poorly understood. Although the expression of ER α is differentially regulated within individual cell types, the role of individual promoters, *cis*-acting elements, transcription factors, ER α itself and the cellular context remain to be elucidated (Reid *et al.* 2002).

In OVX control rats, we observed a constitutive expression of ER α protein in the nucleus of epithelial (glandular and luminal), stromal and myometrial uterine compartments. In accordance with previous studies, E₂ treatment down-regulated the expression of ER α protein in specific cellular types in the rat uterus (Manni *et al.* 1981, Nephew *et al.* 2000). The expression of ER α in epithelium and subepithelial stroma was shown to be very sensitive to estrogen treatment, since the low dose of E₂ used in this study significantly diminished receptor expression. In contrast, the myometrial compartment maintained high levels of nuclear ER α immunostaining even if the animals were treated with the high dose of E₂. Interest-

ingly, the levels of ER α mRNA decreased only when animals received the high dose of E₂, indicating that this steroid can control the ER α transcription–translation cascade at different levels.

An interesting finding from this study is that luminal and glandular epithelium display differential regulation of ER α expression when OVX animals received P₄ alone. While E₂ alone down-regulated ER α protein immunostaining in both epithelial cell types, P₄ induced a selective inhibition of ER α in the luminal epithelial compartment. It should be noted that this P₄-dependent down-regulation of ER α expression was induced without any E₂ priming, demonstrating that the OVX rat uterus is constitutively responsive to P₄. Moreover, we have shown that P₄ action maintained a high expression of ER α in the glandular epithelium. In accordance with our results, Katsuda *et al.* (1999) have found a positive correlation between serum P₄ levels during metestrus and ER α expression in glandular epithelial uterine cells. Moreover, they have reported that a significant increase of bromodeoxyuridine-labeled cells occurred in the uterine glands at metestrus. Therefore, it is necessary to further investigate the role of P₄ in the regulation of ER α in glandular epithelial cells as an indirect mechanism of controlling cell proliferation. Regarding the differential

Figure 4 Expression of ERα mRNA alternative spliced variants in rat uterus detected by RT-nested PCR in adult OVX controls and steroid-treated animals. Abbreviations of experimental groups are detailed in the legend of Fig. 2. (A) Schematic representation of the structure of full-length (FL) ERα mRNA and splicing variants. Σ3, Σ4 and Σ3,4 isoforms were created by exon-skipping mechanisms from the ERα primary transcript. The locations of primers on the cDNA for performing the first (E3, E4) and second (E5, E6) round of nested PCR are shown according to the Materials and Methods and Table 1. (B) The existence of four ERα transcripts in the uterus of controls rats with a differential pattern of expression according to each steroid treatment was observed. The lengths of the amplification products are indicated in the center of the figure. Surprisingly, in rats treated with either the low dose of E₂, P₄ alone or the combination of both steroids, expression of the Σ3 isoform was completely abolished. Assays from two independent RNA samples per experimental group are presented (n=7–8 animals/group).

expression of ERα between luminal and glandular cells during preparation of the uterus for implantation, it has been suggested that these two epithelial cell types respond differentially to P₄ and E₂ action (Tan *et al.* 1999).

The ERα gene constitutes a complex unit exhibiting alternative splicing and promoter usage in a tissue-specific manner (Flouriot *et al.* 1998). A relatively high mRNA turnover rate could be one mechanism, in conjunction with transcription controls, in order to maintain the ERα protein at suitable levels. Our results suggest ERα expression in the uterus of adult rats is dependent upon the dose of E₂ and/or co-treatment with P₄. It is interesting to note that only when animals received the high dose of E₂ or P₄ alone did the uterus exhibit a down-regulation of both ERα protein and ERα mRNA.

Alternative splicing of pre-mRNAs is thought to be one of the cellular mechanisms for generating a functionally diverse pool of gene products derived from a single gene and is also recognized as an important mechanism for regulating wild-type proteins (Poola *et al.* 2002). In this work, we observed that three splice variants of ERα mRNA – exhibiting in-frame deletions of exon 3, exon 4, or both – are present in the uterus of both OVX adult rats and normal cycling females. Moreover, for the first time,

Figure 5 Representative RT-nested PCR assays showing ERα mRNA full length (FL) and the three spliced isoforms (Σ3, Σ4 and Σ3,4) at different stages of the estrous cycle. Animals were killed and uteri collected between 1000 and 1100 h at proestrus (Pro), estrus (Es) or diestrus (Di). Note the very low expression of the Σ3 isoform at diestrus. Assays from two independent RNA samples per experimental group are presented (n=5 animals per stage of estrus cycle).

we observed that alternative splicing of ER α transcripts is influenced by the ovarian steroid hormonal milieu.

The biological relevance of the presence of ER α isoforms in normal tissues is not well established. Their functions may be partially predicted by the structures of those isoforms compared with that of the wild-type ER α (Pasqualini *et al.* 1999). The isoform originating from the deletion of exon 3 was shown to be unable to bind to a canonical ERE (Wang & Miksicek 1991). In the MCF-7 breast cancer cell line, Σ 3 inhibits the activation of estrogen-dependent transcription in a dominant negative fashion when cotransfected with the full-length ER α . It has been suggested that Σ 3 expression in normal tissue may provide a means of decreasing or blocking estrogen responsiveness (Pasqualini *et al.* 2001).

The presence of the Σ 3 isoform may be necessary for regulating the cellular response to estrogens. In our OVX model, those animals that received the high dose of E₂ expressed this splicing isoform, suggesting that Σ 3 could exhibit a 'buffer action' in regulating estrogen responsiveness. This 'buffer action' was observed during pituitary gland ontogeny, allowing regulation of ER α activities by differential splicing of the primary transcript (Pasqualini *et al.* 1999). The physiological relevance of the Σ 3 isoform is suggested by the fact that it is present during the normal estrous cycle. Moreover, Σ 3 isoform mRNA levels are higher during proestrus and estrus when estrogenic effects are maximal. These results support the hypothesis that the Σ 3 isoform may play a significant physiological role that is under steroidal control. A recent report indicates that endogenous estrogen differentially regulates pituitary expression of mRNAs encoding several ER isoforms with distinct functional properties (Tena-Sempere *et al.* 2004). This, together with our current results, allows us to postulate that interaction of ER α protein with its ligand not only activates signaling but also turns on different mechanisms for the fine tuning of uterine responsiveness to estrogen. These would include repression of inhibitory isoforms, which may be essential for the full expression of estrogen effects at the ER α , as well as induction of dominant negative isoforms, which may participate in the auto-limitation of estrogen effects. In addition, since the combination of the high dose of E₂ plus P₄ maintained a high expression of the Σ 3 isoform, this result might suggest that, at least in part, the antagonistic action of P₄ on E₂-mediated effects may be regulated by the presence of the Σ 3 exon-skipping variant. The observation that expression of this variant was not observed with the low dose of E₂ suggests that the ligand-mediated regulation of ER α exon-skipping variant expression may be dose dependent. It has been demonstrated that steroid receptor isoforms are differentially expressed depending on the type of tissue examined and the anatomical localization (Guerra-Araiza *et al.* 2003, Patchev *et al.* 2004). Moreover, the splicing isoforms of ER α are distributed in different subcellular compartments (Pasqualini *et al.* 1999). These

results, taken together, strongly support the hypothesis that differential splicing of the ER α transcripts may be an extensive mechanism for controlling ER α -mediated cellular responses.

In summary, this is the first time that the presence of the ER α Σ 3 exon-skipping variant has been shown to be regulated by E₂ and P₄ in the rat uterus. The data presented here suggest that this regulation is dose dependent and could be a regulatory mechanism of E₂ action. Actually, we are evaluating ER α mRNA isoforms in each individual cellular compartment of the rat uterus, using a combination of laser capture microdissection and nested real time-PCR. This technology could allow us to better understand the complex regulation of ER α mRNA differential splicing in each individual cell type. Further characterization of this receptor isoform may increase the understanding of the mechanisms of action of therapeutic and environmentally relevant estrogen-like compounds.

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