Neonatal exposure to estrogen differentially alters estrogen receptor α and β mRNA expression in rat testis during postnatal development

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Abstract

The biological actions of estrogens on target cells are mediated by two nuclear receptors: the estrogen receptor (ER) α and the recently characterized ER β . In the male rat, the physiological role of estrogens involves multiple actions, from masculinization of brain areas related to reproductive function and sexual behavior to regulation of testicular development and function. Paradoxically, however, administration of high doses of estrogen during the critical period of neonatal differentiation results in an array of defects in the reproductive axis that permanently disrupt male fertility. The focus of this study was to characterize the effects and mechanism(s) of action of neonatal estrogenization on the pattern of testicular ER α and β gene expression during postnatal development. To this end, groups of male rats were treated at day 1 of age with estradiol benzoate (500 μ g/rat), and testicular ER α and ER β mRNA levels were assayed by semi-quantitative RT-PCR from the neonatal period until puberty (days 1–45 of age). Furthermore, the expression of androgen receptor (AR) mRNA was evaluated, given the partially overlapping pattern of tissue distribution of ER α , ER β

and AR messages in the developing rat testis. In addition, potential mechanisms for neonatal estrogen action were explored. Thus, to discriminate between direct effects and indirect actions through estrogen-induced suppression of serum gonadotropins, the effects of neonatal estrogenization were compared with those induced by blockade of gonadotropin secretion with a potent LHRH antagonist in the neonatal period. Our results indicate that neonatal exposure to estrogen differentially alters testicular expression of α and β ER messages: ER α mRNA levels, as well as those of AR, were significantly decreased, whereas relative and total expression levels of ER β mRNA increased during postnatal/prepubertal development after neonatal estrogen exposure, a phenomenon that was not mimicked by LHRH antagonist treatment. It is concluded that the effect of estrogen on the expression levels of ER α and β mRNAs probably involves a direct action on the developing testis, and cannot be attributed to estrogen-induced suppression of gonadotropin secretion during the neonatal period.

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Introduction

Despite the classical contention that estrogen is the female hormone, compelling evidence indicates a pivotal role of estrogen in the regulation of development and function of several systems in the male. In mammals, these actions include permanent masculinization of brain structures involved in neuroendocrine control of gonadal function and reproductive behavior (MacLusky & Naftolin 1981, Arnold & Gorski 1984), regulation of differentiation and function of Leydig cells within the testis (Brinkmann *et al.* 1980, Abney & Myers 1991), modulation of luminal fluid resorption in the epididymis (Hess *et al.* 1997), and control of follicle-stimulating hormone (FSH) secretion (Sharpe

1998), as well as direct effects on bone and the cardiovascular system (Nilsson *et al.* 1998, Sharpe 1998).

The biological actions of estrogen are targeted through interaction with a specific intranuclear receptor, termed the estrogen receptor (ER). Two different subtypes of ER have been identified so far: the classical ER (renamed ER α), and the recently characterized ER β (Green *et al.* 1986, Greene *et al.* 1986, Koike *et al.* 1987, Kuiper *et al.* 1996, Tremblay *et al.* 1997). Interestingly, α and β forms of ER have been shown to possess different binding affinities for a variety of estrogenic compounds (Kuiper *et al.* 1997), distinct patterns of tissue distribution (Fisher *et al.* 1997, Shughrue *et al.* 1997, Mitchner *et al.* 1998, Österlund *et al.* 1998, Saunders *et al.* 1998, Sar & Welsch 1999), as well as

different transactivation activities on estrogen-regulated gene constructs (Paech *et al.* 1997, Pennie *et al.* 1998). Indeed, identification of the novel ER β has attracted considerable attention as it may provide the molecular basis for the complexity and plasticity of biological actions of naturally occurring and synthetic estrogenic compounds (Kuiper *et al.* 1998).

In male rodents, it is well established that exposure to supra-physiological doses of estrogens during the critical period of neonatal differentiation results in an array of permanent reproductive defects, which include atrophy of testes and sexual accessory glands (Kincl *et al.* 1965, Brown-Grant *et al.* 1975, Aguilar *et al.* 1984, Newbold & McLachlan 1985, Naslund & Coffey 1986), impaired maturation of germ, Sertoli and Leydig cells (Gaytán & Aguilar 1986, Gaytán *et al.* 1986, Pinilla *et al.* 1992, Sharpe *et al.* 1998), altered luteinizing hormone (LH) secretion (Aguilar *et al.* 1984, Pinilla *et al.* 1995), and inappropriate puberty onset (Brown-Grant *et al.* 1975, Bellido *et al.* 1985). Disruption of reproductive function by neonatal estrogenization is probably a multifaceted phenomenon, and complete elucidation of the mechanism(s) involved is still pending. Concerning testicular damage, experimental evidence has emphasized the role of estrogen-induced decrease in gonadotropin secretion neonatally (Bellido *et al.* 1990), although a direct action of estrogens on the developing male gonad has been suggested recently (Sharpe *et al.* 1998). Worthy of note, little attention has been paid to molecular events in the rodent testis after neonatal estrogenization, although analogous studies have been carried out on the prostate and uterus (Pylkkanen *et al.* 1993, Prins & Birch 1995, Zheng & Hendry 1997, Prins *et al.* 1998). Interestingly, complete characterization of the deleterious effects and the mechanism(s) of action of exogenous estrogens, administered during the fetal/neonatal period, on differentiation and function of reproductive organs has become relevant in the face of potential hazardous effects of environmental xenoestrogens on male fertility (for a review see Toppari *et al.* 1996).

In the present study, we aimed at evaluating the effect of neonatal estrogen exposure on the pattern of ER α and ER β mRNA expression in rat testis during development from birth to puberty. Further, the impact of neonatal estrogenization on the pattern of expression of androgen receptor (AR) mRNA was analyzed, given the partially overlapping pattern of cellular distribution of ER α , ER β and AR messages in developing rat testis (Bremner *et al.* 1995, Fisher *et al.* 1997, Saunders *et al.* 1998, Van Pelt *et al.* 1999). In addition, we sought potential mechanisms underlying the effects of neonatal administration of estrogen on testicular ER α and ER β mRNA expression. Thus, considering that the developing testis has been suggested as a potential target for estrogen action (Fisher *et al.* 1997, Saunders *et al.* 1998, Sharpe *et al.* 1998, Van Pelt *et al.* 1999), we explored the possibility of a direct action of estrogen at the testicular level by comparing the

effects of neonatal estrogenization with those induced by the blockade of gonadotropin secretion through administration of a potent LH-releasing hormone (LHRH) antagonist (LHRH-ANT) neonatally.

Materials and Methods

Animals and drugs

Male Wistar rats bred in the vivarium of our institution were used. The day the litters were born was considered day 1 of life. At this time, the litter size was adjusted to eight rats per dam. The animals were maintained under controlled conditions of light (14 h of light; lights on at 0700 h) and temperature (22 °C). The animals were weaned at 21 days of age and housed thereafter in groups of five per cage, with free access to pelleted food and tap water.

Estradiol benzoate (EB) was obtained from Sigma (St Louis, MO, USA), and the LHRH-ANT Org 30276 (Ac-D-pClPhe-D-pClPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH₂CH₃COOH) was generously supplied by Organon (Oss, Netherlands). For administration, EB was dissolved in olive oil and the LHRH-ANT in physiological saline (0.9% NaCl).

Experimental designs

The effects of neonatal exposure to estrogen on the pattern of ER α , ER β and AR mRNA expression during postnatal testicular development were monitored in Experiment 1. To this end, 1-day-old male rats were injected s.c. with a single dose of EB (500 µg/rat); this protocol was selected on the basis of our previous studies as it was found to be the most effective in inducing complete estrogenization in the male rat (Aguilar *et al.* 1984, Bellido *et al.* 1985, 1990, Gaytán & Aguilar 1986, Gaytán *et al.* 1986, Pinilla *et al.* 1992, 1995). Vehicle (oil)-injected rats served as controls. Groups of animals were sequentially killed on day 1 (within the first 24 h of postnatal life, i.e. before EB administration), and on days 5, 10, 15, 20, 30 and 45 of age. In Experiment 2, the potential mechanism(s) involved in the effects of neonatal estrogenization on the pattern of testicular ER α , ER β and AR mRNA expression was evaluated. Since the effects of neonatal exposure to estrogen on the reproductive axis are mediated, at least partially, by postnatal suppression of gonadotropins (Bellido *et al.* 1990), expression levels of ER α , ER β and AR mRNAs were evaluated in testes of rats treated neonatally with LHRH-ANT, and compared with those of neonatally estrogenized males. Male rats were injected s.c. with LHRH-ANT (5 mg/kg body weight) on days 1, 4, 7, 10, 13 and 15 of age, as described previously (Pinilla *et al.* 1994). For comparative analysis, two age-points were selected for tissue sampling: day 15 (4 h after the last

LHRH-ANT injection) and day 45. In all experiments, trunk blood, testes, and ventral prostates were taken, and the weights of the organs recorded. Sera were separated from blood samples and stored at -20°C until used for hormone measurements. After removal, the testes were immediately frozen in liquid nitrogen and stored at -70°C until used for RNA analyses. All the experimental procedures were approved by the Córdoba University Committee on Laboratory Animal Care, and were conducted in accordance with the European Union Normative for care and use of experimental animals.

RNA analysis by semi-quantitative RT-PCR

RT-PCR, optimized for semi-quantitative detection (see below), was used to analyze relative expression levels of ER α , ER β and AR mRNAs in the different experimental groups. Total RNA was isolated from testicular samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method, as described previously (Chomczynski & Sacchi 1987). For amplification of the target genes, the following primer pairs were used: ER α -sense (nt 472–492; 5'-AAT TCT GAC AAT CGA CGC CAG-3') and ER α -as (nt 816–794; 5'-GTG CTT CAA CAT TCT CCC TCC TC-3'), for amplification of a 344-bp fragment of rat ER α cDNA; ER β -sense (nt 38–58; 5'-TTC CCG GCA GCA CCA GTA ACC-3') and ER β -as (nt 299–279; 5'-TCC CTC TTT GCG TTT GGA CTA-3'), for amplification of a 262-bp fragment of rat ER β cDNA; and AR-sense (nt 1623–1645; 5'-CCC ATC GAC TAT TAC TTC CCA CC-3') and AR-as (nt 1913–1891; 5'-TTC TCC TTC TTC CTG TAG TTT GA-3'), for amplification of a 291-bp fragment of AR cDNA. The reported sets of primers were selected based on previous references (Shan *et al.* 1995, Kuiper *et al.* 1997), and synthesized according to the published cDNA sequences of rat ER α (Koike *et al.* 1987), ER β (Kuiper *et al.* 1996) and AR (Chang *et al.* 1988). In addition, to provide an appropriate internal control, coamplification of a 290-bp fragment of the L19 ribosomal protein mRNA was carried out in each sample using the primer pair: L19-sense (nt 91–110; 5'-GAA ATC GCC AAT GCC AAC TC-3') and L19-as (nt 380–361; 5'-ACC TTC AGG TAC AGG CTG TG-3'), generated according to the rat L19 ribosomal protein cDNA (Chang *et al.* 1987).

For amplification of the target genes, RT and PCR were run in two separate steps. Furthermore, to enable appropriate amplification in the exponential phase for each target, PCR amplification of ER α , ER β , AR and L19 ribosomal protein transcripts was carried out in separate reactions with a different number of cycles (see below), but using similar amounts of the corresponding cDNA templates, generated in single RT reactions, as described elsewhere (Santana *et al.* 1996, Tena-Sempere *et al.* 1999). Briefly, equal amounts of total testicular RNA (4 μg) were heat denatured and reverse transcribed by incubation at

42°C for 90 min with 12.5 U avian myeloblastosis virus (AMV) RT (Promega, Madison, WI, USA), 20 U ribonuclease inhibitor RNasin (Promega), 200 nM deoxy-NTP mixture, and 200 pmol random hexamer primers (Promega), in a final volume of 30 μl 1 $\times$ AMV-RT buffer. The reactions were terminated by heating at 97°C for 5 min and cooling on ice, followed by dilution of the RT cDNA samples with nuclease-free H_2O (final volume 60 μl). For semi-quantitative PCR, 10 μl aliquots of the cDNA samples (equivalent to 650 ng total RNA input) were amplified in 50 μl 1 $\times$ PCR buffer in the presence of 2.5 U Taq-DNA polymerase (Promega), 200 nM deoxy-NTP mixture, and the appropriate primer pairs (1 nM of each primer; see above). PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 15 min was included. The number of cycles was optimized to ensure amplification in the exponential phase of PCR. Different number of cycles were tested for ER α , ER β , AR (ranging between 25 and 45) and L19 ribosomal protein (ranging between 14 and 30) and 33, 37, 35 and 20 cycles, respectively, were chosen for further analysis (see Results).

The generated cDNA fragments were resolved in 1.5% agarose gels containing ethidium bromide (0.1 $\mu\text{g}/\text{ml}$), and their molecular sizes determined by comparison with size markers (PCR 50-bp Step Ladder, Promega) run together with the cDNA products (1-D Manager software, TDI Ltd, Madrid, Spain). Specificity of PCR products was confirmed by Southern hybridization, using radiolabeled nested oligonucleotide primers (data not shown), as described elsewhere (Kuiper *et al.* 1997, Tena-Sempere *et al.* 1999). For quantitative evaluation of RT-PCR signals, electrophoretically separated cDNA fragments were photographed using 667 Polaroid films (Polaroid, Cambridge, MA, USA), and the images were digitized using an IBM flatbed scanner. Absolute optical densities (ODs) for each PCR product were obtained by densitometric scanning of the digital signals using an image analysis system (1-D Manager, TDI Ltd), and the values for the specific target genes (ER α , ER β and AR) were normalized with respect to those of L19 ribosomal protein to express arbitrary units of relative abundance of the specific messages (relative expression). In addition, as neonatal administration of EB and LHRH-ANT induced permanent atrophy of testes, semi-quantitative presentation of densitometric values after correction for testicular weight was also included. This gives a reliable estimate of total level of expression of each message per testis (total expression). To ensure that equal inputs of RNA were added to RT-PCR reactions, only samples yielding roughly similar OD values for L19 bands were considered for further analysis. In addition, to minimize potential RT-PCR artifacts due to inherent reaction variability, all

data-points were repeated, for each target gene, at least three times using independent RNA samples. Finally, primer pairs were synthesized according to previous references (Shan *et al.* 1995, Kuiper *et al.* 1997), designed to span over intronic sequences to exclude potential amplification of contaminant genomic DNA. Furthermore, in all assays, liquid controls and reactions without RT were included, yielding negative amplification (data not shown).

Hormone measurements

Serum LH levels were measured using a supersensitive, time-resolved, immunofluorometric assay (IFMA-Delfia, Wallac Oy, Turku, Finland), as described elsewhere (Haavisto *et al.* 1993). The results were expressed in terms of the reference preparation LH-RP-2 (National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA); the sensitivity of the assay was 0.75 pg/tube, the intraassay coefficient of variation (CV) was 7%, and the interassay CV 10%. Serum FSH levels were assayed by a novel time-resolved immunofluorometric assay, based on the Delfia principle (Hakola *et al.* 1999), with minor modifications. A new pair of antibodies was used: an anti-human FSH β monoclonal antibody (FSH 56 A) as the capture antibody, and a polyclonal anti-human α -subunit antibody (R93–2705) as the signal antibody. Both were kindly donated by Dr W Schoonen (Organon). The results were expressed in terms of the reference preparation FSH-RP-2 (NIDDK); the analytical sensitivity of the assay was 203 pg/tube, the intraassay CV was <4%, and the interassay CV was 5.5%. Serum testosterone levels were measured by RIA, after diethyl ether extraction of the samples, using 125 I-labeled testosterone (Orion-Farmos Diagnostica, Turku, Finland) as tracer and testosterone antiserum kindly donated by Dr R Vihko (Department of Clinical Chemistry, Oulu University, Finland).

Statistics

Semi-quantitative data are expressed as means $\pm$ S.E.M. from three to five independent observations. Statistically significant differences between groups were determined by one-way ANOVA, followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Endocrine profiles (Fig. 1)

To evaluate the endocrine background for testicular expression of ER α , ER β and AR mRNAs, serum FSH, LH and testosterone levels were measured, and testicular weights were recorded, in the experimental groups.

Figure 1 Testicular weights (upper panel), and serum FSH, LH and testosterone (T) levels (middle and lower panels) in the experimental groups indicated, at different time-points along the developmental time-frame between the neonatal period (day 1 of age) and puberty (day 45 of age). The experimental groups were: control rats, rats treated neonatally with EB (500 μ g/rat, on day 1), and rats treated with LHRH-ANT (LHRH-A) during the neonatal period (5 mg/kg every 72 h, from days 1 to 15). Values are given as means $\pm$ S.E.M. ($n = 4$ –8 samples/group). ** $P < 0.01$ vs values from corresponding controls; a= $P < 0.01$ vs values from corresponding EB-injected groups (ANOVA followed by Tukey's test).

Control animals showed a gradual increase of testicular weight along the study period (from days 1 to 45 of age). In this group, low levels of FSH and LH were detected from days 1 to 20, followed by a sharp elevation between days 20 and 30 of age. Serum testosterone values were low at birth, increased gradually from days 1 to 15 of age, and remained similar thereafter. However, a further increase in

serum testosterone levels took place in paired, 75-day-old male rats (1800.6 ± 391.5 ng/l; personal observation). In keeping with previous references (Kincl *et al.* 1965, Brown-Grant *et al.* 1975, Bellido *et al.* 1985, 1990, Pinilla *et al.* 1992, 1995), neonatal administration of EB (500 μ g/rat; day 1) resulted in permanent atrophy of testes, and significantly decreased serum FSH, LH and testosterone levels during the study period. Finally, neonatal administration of LHRH-ANT (5 mg/kg every 72 h; days 1–15) induced a significant reduction in testicular weight on days 15 and 45 of age, as well as significant decreases in serum FSH, LH and testosterone values on day 15 of age. However, in line with previous reports (Huhtaniemi *et al.* 1986, Pinilla *et al.* 1994), serum testosterone concentrations were normal, and FSH and LH levels were increased over control values in 45-day-old animals neonatally treated with LHRH-ANT. Based on previous references, such a rebound in serum gonadotropin levels was probably initiated around day 25 of age (Pinilla *et al.* 1994).

Optimization of semi-quantitative RT-PCR assays

RT-PCR assays were optimized for semi-quantitative analysis of ER α , ER β , AR and L19 ribosomal protein mRNA expression. To obtain optimal conditions for amplification, i.e. in the exponential phase of PCR, different numbers of PCR cycles were tested for each message. As shown in Fig. 2, plotting of intensity of PCR signals (as expressed by absolute OD values) against the number of amplification cycles revealed a strong linear relationship between cycles 28 and 36 in the case of ER α (correlation coefficient $r^2=0.9601$), cycles 30 and 38 in the case of ER β ($r^2=0.9639$), cycles 30 and 36 in the case of AR ($r^2=0.9761$), and cycles 14 and 23 in the case of L19 ribosomal protein ($r^2=0.9980$). For this reason, PCR amplification of ER α , ER β , AR and L19 ribosomal protein transcripts was carried out in separate reactions, using 33, 37, 35 and 20 amplification cycles respectively. The validity of the above RT-PCR assays for semi-quantitative evaluation is supported by the following facts. First, single RT reactions using random hexamers were carried out for each sample, and similar amounts of generated cDNA (equivalent to an approximate input of 650 ng total RNA per sample) were used for further PCR amplification of each target gene. Secondly, only samples yielding similar OD values for L19 ribosomal protein transcripts were considered for analysis and, for semi-quantitative presentation of data, expression values of specific target genes (ER α , ER β and AR) were normalized with respect to those of L19 ribosomal protein. Thirdly, all data-points were repeated, for each target gene, at least three times using independent RNA samples, thus avoiding potential RT-PCR artifacts due to inherent reaction variability. Finally, when the selected RT-PCR conditions were tested with increasing amounts

of total RNA, a linear relationship between intensity of PCR signals and amount of starting RNA was found (Fig. 3).

Testicular expression of ER α , ER β and AR mRNAs in neonatally estrogenized rats

Testicular expression of ER α , ER β and AR mRNAs was monitored in control and neonatally estrogenized animals throughout postnatal development by means of semi-quantitative RT-PCR. Representative expression patterns of ER α , ER β , AR and L19 mRNAs at different age-points from the neonatal period until puberty (days 1–45 of age) are presented in Figs 4 and 5. A summary of semi-quantitative data on the steady-state ER α , ER β and AR mRNA levels throughout the study period is shown in Fig. 6. For clear presentation of data on relative expression (Fig. 6, left panels), the level of each specific message (ER α , ER β and AR) on day 1 of age was taken as 100% and the other values were normalized accordingly, thus allowing a semi-quantitative comparison between different time-points. In control animals, high levels of expression of ER α , ER β and AR mRNAs were detected in testes from neonatal-infantile rats. In the case of ER α and AR messages, a moderate decrease in the level of relative expression was observed from days 10 to 15 of age onwards, the values in 45-day-old rats being, respectively, between 60 and 65% and 70 and 75% of maximum levels in the infantile period. In contrast, a sharp decrease in the relative level of expression of ER β mRNA was noted between days 15 and 20 of age. From this time-point onwards, ER β mRNA levels remained low, ranging between 5 and 15% of maximum values of the neonatal period. Neonatal estrogenization profoundly altered the pattern of testicular expression of ER α , ER β and AR mRNAs throughout the study period. Both relative levels and total expression of ER α and AR mRNAs decreased in testes from rats neonatally treated with estrogen; the reduction in relative mRNA levels of ER α and AR was statistically significant from day 15 onwards, but from days 5–10 onwards when considered in terms of total expression per testis. On the contrary, neonatal exposure to estrogen induced a marked increase in both relative levels and total expression of ER β mRNA. This increase was statistically significant at all age-points studied.

Testicular expression of ER α , ER β and AR mRNAs in neonatally LHRH-ANT-treated rats

Representative expression patterns of ER α , ER β , AR and L19 mRNAs in testes from 15- and 45-day-old rats treated neonatally with LHRH-ANT are shown in Fig. 5. In addition, compilation of semi-quantitative data on the steady-state levels of these messages is included in Fig. 6. Administration of LHRH-ANT during the neonatal

Figure 2 Optimization of RT-PCR conditions for semi-quantitative determination of target mRNAs. For amplification in the exponential phase of PCR, different numbers of cycles were tested for each message. Quantitative analysis of the cycle dependency for the generated PCR signals revealed a strong linear relationship between cycles 28 and 36 in the case of ER α (correlation coefficient $r^2=0.9601$), cycles 30 and 38 in the case of ER β ($r^2=0.9639$), cycles 30 and 36 in the case of AR ($r^2=0.9761$), and cycles 14 and 23 in the case of L19 ribosomal protein ($r^2=0.9980$). Optimization of RT-PCR conditions was carried out using total RNA from control, 10-day-old rat testis. Values are given as means $\pm$ s.d. of two independent determinations. A representative ethidium bromide-stained gel electrophoresis of the DNA products generated for each target is presented in the insets.

period failed to significantly alter the pattern of relative expression of ER α , ER β and AR mRNAs in testes from 15-day-old rats, and only a slight increase, which was not significant, of relative expression levels of ER β mRNA was apparent at this age-point. In addition, a significant

decrease in terms of total expression was detected for ER α . On day 45 postpartum, relative levels as well as total expression per testis of ER α and AR mRNAs were significantly decreased in animals neonatally treated with LHRH-ANT, whereas those of ER β were at the

Figure 3 Dose dependency of the intensity of PCR signals. Increasing amounts of total RNA from control, 10-day-old rat testis were amplified by RT-PCR, using conditions selected for analysis of experimental samples (see Results). Quantitative analysis revealed a strong linear relationship between the amount of starting RNA and the intensity of the generated PCR signals, for each target message, under the selected conditions. Values are given as means $\pm$ S.D. of two independent determinations. A representative ethidium bromide-stained gel electrophoresis of the DNA products for each target is presented in the insets.

limit of detection and not significantly different from their respective controls.

Discussion

To our knowledge, this is the first study to report the effects of neonatal exposure to estrogen on the expression of ER α and ER β mRNAs in rat testis during postnatal/prepubertal development. In this sense, although alterations

in testicular morphology and function after neonatal exposure to estrogenic compounds have been well characterized (as examples see Gaytan & Aguilar 1986, Gaytan *et al.* 1986, Tena-Sempere *et al.* 1993, Sharpe *et al.* 1998), little attention has been paid to molecular events in the rat testis after neonatal estrogenization. Several reasons prompted us to look for changes in the level of testicular expression of ER α and ER β genes in this experimental model. First, the presence of ER α and ER β in the developing testis has been well documented (Fisher *et al.*

Figure 4 Representative ethidium bromide-stained gel electrophoresis of ER α , ER β , AR and L19 cDNA fragments amplified by RT-PCR, using conditions selected for semi-quantitative analysis. Assessment of the level of expression of the target genes was carried out from total testicular RNA samples of control (C) and neonatally estrogenized (EB) rats, at different time-points between days 1 and 30 of age. An additional age-point (day 45) is presented in Fig. 5. The sizes of the generated products were calculated by comparison with the mobility of size markers (PCR 50-bp Step Ladder; data not shown), and are indicated on the right.

1997, Saunders *et al.* 1998, Van Pelt *et al.* 1999), yet little was known about the homologous regulation of their expression at the mRNA level. Secondly, the molecular phenotype and mechanism(s) of testicular damage in the neonatally estrogenized rat remained to be fully characterized, and no attempt had been made to evaluate the potential contribution of changes in the pattern of expression of ER subtypes. In addition, identification of specific responses in terms of ER β gene expression may help to elucidate its role, if any, in testicular development and function. In this sense, the widespread expression of ER β in the male gonad, as well as its distinct pattern of cellular distribution from ER α (Fisher *et al.* 1997, Saunders *et al.* 1998, Van Pelt *et al.* 1999), strongly suggested a specific role of the β form of ER in the regulation of testicular function. However, as reported recently (Krege *et al.* 1998), targeted disruption of ER β gene failed to cause overt abnormalities in the reproductive tract, and neither did it impair male fertility. This is in contrast to the reproductive phenotype of knockout mice lacking the genes encoding ER α (Eddy *et al.* 1996) or aromatase (Robertson *et al.* 1999). The possibility remains, however, that although not absolutely essential for reproduction in

the male, the β form of ER may play a role in the fine-tuning of estrogen actions on testicular function. Interestingly, redundant regulatory pathways seem to operate within the testis to ensure fertility; as an example, the lack of biological actions of FSH, despite its well-proven role in the regulation of spermatogenesis, is associated with partially preserved male fertility, in both humans and rodents (Kumar *et al.* 1997, Tapanainen *et al.* 1997).

In our study, assessment of the level of expression of ER α and ER β mRNAs was carried out by means of RT-PCR, optimized for semi-quantitative detection. Due to its high sensitivity and the ability to detect alternatively spliced transcripts, this method has become widely used for the analysis of ER β mRNA expression in a variety of tissues, including the testis (for examples see Kuiper *et al.* 1997, Karolczak & Beyer 1998, Petersen *et al.* 1998). In our approach, the reliability of semi-quantitative results is supported by (i) the selection, for each target gene, of amplification conditions in the exponential phase of PCR (Fig. 2), (ii) the repetitive observation of results for different RNA samples within the same experimental group, (iii) the use of an appropriate internal control and (iv) the positive linear correlation between RNA input and intensity of signal, using the selected RT-PCR conditions (Fig. 3). Semi-quantitative comparison was carried out in terms of relative mRNA levels and total expression per testis (i.e. after correction of densitometric values for testicular weight). The former gives an estimate of the relative abundance of each specific message, whereas the latter provides information on their total level of expression per gonad. This is relevant considering that neonatal administration of EB and LHRH-ANT induced a permanent reduction in testicular weight during the study period (Fig. 1).

Semi-quantitative evaluation by RT-PCR revealed that testicular expression of ER α and ER β mRNAs is imprinted by neonatal exposure to estrogen in a strikingly opposite manner. Overall, relative levels and total expression of ER α mRNA were decreased in neonatally estrogenized rats during postnatal/prepubertal testicular development. On the contrary, testicular ER β expression, in terms of both relative levels and total RNA expression, was increased in this animal model during the developmental time-frame studied. Moreover, neonatal estrogenization differentially altered the pattern of testicular expression of ER β and AR mRNAs, as an overall reduction was observed in the relative levels and total RNA expression of AR, despite the fact that expression of both genes shares, at least partially, the pattern of cellular distribution within the testis (Bremner *et al.* 1995, Saunders *et al.* 1998, Van Pelt *et al.* 1999).

The mechanism(s) of action of neonatal estrogen on the developing testis was approached by comparing its effects with those induced by the blockade of gonadotropin secretion through administration of a potent LHRH-ANT during the neonatal period. This comparative setting has

Figure 5 Representative ethidium bromide-stained gel electrophoresis of ER α , ER β , AR and L19 cDNA fragments amplified by RT-PCR, from total testicular RNA samples of controls (C), neonatally estrogenized (EB) rats, and rats treated neonatally with LHRH-ANT (A). For comparison, two age-points are presented: day 15 (4 h after the last LHRH-ANT injection) and day 45. The sizes of the generated products were calculated by comparison with the mobility of size markers (PCR 50-bp Step Ladder; data not shown), and are indicated on the right.

proven helpful in discriminating between direct actions and indirect effects of neonatal estrogenic compounds on the developing testis (Sharpe *et al.* 1998). Such an analysis revealed that the changes in testicular expression of ER α , ER β and AR messages were not solely attributable to estrogen-induced decrease in gonadotropin secretion during the critical period of neonatal differentiation, as relative levels of expression of ER α , ER β and AR mRNAs in 15-day-old rat testes were not significantly affected by LHRH-ANT treatment, and neither were those of ER β in 45-day-old rats neonatally treated with LHRH-ANT. Moreover, total expression per testis was significantly different between neonatally estrogenized and LHRH-ANT-treated groups for ER α (on day 45 of age), ER β and AR (on days 15 and 45 of age) mRNAs. As a whole, our data suggest a direct action of estrogen on the developing testis and indicate divergences in the mechanisms for the effects of neonatal estrogenization on the morphological development of the testis and the pattern of expression of ER α , ER β and AR messages. On the

former, previous experimental work has clearly demonstrated that estrogen-induced decrease in gonadotropin secretion during the neonatal period is the major cause for testicular atrophy in neonatally estrogenized rats (Bellido *et al.* 1990). Alternatively, as LHRH receptors have been identified in the neonatal rat testis (Huhtaniemi *et al.* 1984), we cannot rule out the possibility that, at least partially, the effects of neonatal administration of LHRH-ANT might be due to a direct action at the testicular level. However, the apparent limited relevance of locally produced LHRH in the control of testicular function (Huhtaniemi *et al.* 1987) casts doubts on such a possibility.

Interpretation of the above results is simplified by previous references showing the pattern of gene expression and cellular distribution of ER α , ER β and AR within the developing rat testis. In this sense, it is well documented that expression of ER α is confined to Leydig cells (Fisher *et al.* 1997), whereas ER β expression, at protein and mRNA levels, has been located to several types of germ cell (including gonocytes, type A spermatogonia,

Figure 6 Compilation of semi-quantitative data on the steady-state levels of ER α , ER β and AR mRNAs in testes from controls, neonatally estrogenized (EB) rats, and rats treated neonatally with LHRH-ANT (LHRH-A), at different time-points between the neonatal period (day 1 of age) and puberty (day 45 of age). Relative levels of expression were obtained, in each sample, by normalization of absolute ODs of each target (ER α , ER β and AR) with respect to that of the L19 signal, to express arbitrary units of relative abundance of the specific messages (left panels). Total levels of expression per testis were obtained after correction of relative values for testicular weight (OD·mg TW; right panels). Note that the latter values are plotted using semi-logarithmic presentation. Values are given as means $\pm$ S.E.M. of at least three independent determinations. ** $P < 0.01$ vs values from corresponding controls; a= $P < 0.01$ vs values from corresponding EB-injected groups (ANOVA followed by Tukey's test).

some pachytene spermatocytes and round spermatids) and Sertoli cells, as well as, at low levels, in fetal Leydig cells (Saunders *et al.* 1998, Van Pelt *et al.* 1999). In addition, prominent expression of AR has been identified in Sertoli cells during prepubertal development, a period when ARs are also abundant in peritubular myoid cells and interstitial cells (Bremner *et al.* 1995). On this basis, the decrease in the relative levels and total expression of ER α and AR mRNAs could be attributable, at least partially, to impaired development of testicular cell types expressing these genes after neonatal estrogenization (Gaytan & Aguilar 1986, Gaytan *et al.* 1986, Pinilla *et al.* 1992). However, the increase in the expression of ER β mRNA

in this experimental model cannot be explained through alterations in the cellular composition of the testis, as it is well established that neonatal exposure to estrogen impairs/delays maturation of germ, Sertoli and Leydig cells within the developing male gonad (Gaytan & Aguilar 1986, Gaytan *et al.* 1986, Pinilla *et al.* 1992). Furthermore, although alterations in cellular composition of testes (i.e. spermatogenic arrest with degenerating germ cells and delayed maturation of Sertoli and Leydig cells) are roughly similar between neonatally estrogenized and LHRH-ANT-treated male rats (Gaytan *et al.* 1986, Pinilla *et al.* 1994), striking differences were found in the pattern of response to these experimental manipulations in terms of

ER β mRNA expression, as significant increases in both relative levels and total mRNA expression were found only in estrogen-treated groups. Moreover, the possibility that the latter could be merely due to relative 'enrichment' of ER β message after testicular shrinkage can be ruled out as neonatal estrogenization and LHRH-ANT treatment induced similar reduction in testis weight on day 15 of age (Fig. 1), yet only the former induces a significant increase in ER β mRNA expression at this time-point. Taken together, our results strongly suggest that neonatal estrogen administration results in increased ER β mRNA levels by acting directly on the developing testis. In addition, the possibility that changes in gonadotropin secretion after neonatal estrogen administration may contribute, at least partially, to its reported effects on ER α and β mRNA expression cannot be completely excluded. In this scenario, identification of the pattern of cellular expression underlying the differential responses to neonatal estrogen administration in terms of ER α and ER β mRNA levels will be our obvious next step.

In conclusion, the data presented herein demonstrate that neonatal exposure to estrogen differentially alters testicular expression of ER α and ER β mRNAs during postnatal/prepubertal development. The mechanism for such a phenomenon is probably a direct action of estrogen on the developing testis, and is not attributable to estrogen-induced suppression of gonadotropin secretion during the neonatal period.

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