Ontogenesis of LH and FSH receptors in postnatal rabbit testes: age-dependent differential expression of long and short RNA transcripts

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The ontogeny of testicular LH and FSH receptors was studied in New Zealand rabbits from 20 to 180 days postpartum. The concentrations of free receptors (per mg total proteins) were very low at day 20. They increased steeply at day 30 for the LH receptor and at day 50 for the FSH receptor. Three RNA bands (1.2, 2.5 and 3 kb) were repeatedly detected on northern blots for the LH receptor and two bands (1.2 and 2.2 kb) were detected for the FSH receptor. The 1.2 kb band (which cannot give rise to full-length, membrane-anchored receptor) was present throughout the 20–180 day period for each receptor. However, the higher molecular mass bands were nearly undetectable at day 20. The 2.5 and 3 kb bands of the LH receptor increased twofold between day 20 and day 120, while the 2.2 kb band of the FSH receptor increased fivefold between day 20 and day 75. Thus the very low concentrations, or even absence, of the larger transcripts of both LH and FSH receptors were correlated with the inability to detect their cognate protein until 20 days of age. Subsequently, coordinated increases in high molecular mass transcripts and protein were observed for both receptors. Total LH receptor content increased in parallel to the previously reported increase in plasma testosterone between day 65 and day 100. FSH receptor density began to increase steeply at day 50, just at the onset of spermatogenesis. Thus, postnatal testicular development in the rabbit seems to entail the transcription of high molecular mass, translatable transcripts of the gonadotrophin receptors.

Introduction

Rabbit testes undergo both morphological and functional changes at the onset of puberty, starting at around 45 days of age with the first gonadal mitosis. Between 42 and 140 days, the mass of the testis increases from 0.5 g to about 6 g. At day 50–60, the onset of spermatogenesis is indicated by the appearance of spermatogonia A and B. Shortly thereafter, plasma concentrations of testosterone increase steeply from day 65 until day 100, when the first spermatids appear (reviewed in Boussit, 1989). This suggests that during this period of sexual development, somatic cells (particularly Leydig and Sertoli cells) involved in testis physiology increase their number and metabolism.

Since the activity of these cells is largely controlled by gonadotrophins (that is, Leydig cells by LH and Sertoli cells by FSH), it was considered useful to study the expression of the gonadotrophin receptors during this period. Thus, the testicular content of LH and FSH receptors was evaluated by radio-receptor assay and the size and abundance of their mRNAs

were analysed by northern blotting in rabbits between 20 and 180 days of age.

Materials and Methods

Animals

New Zealand male rabbits (10–24 of each age group) were reared at INRA local facilities in Jouy-en-Josas. Animals were killed; testes were dissected out, snap frozen in liquid nitrogen and then stored at -80°C . Testis mass, with tunica albuginea, but devoid of annexes (epididymis, tunica vaginalis) was measured before freezing and was about 25 mg at day 20, 65 mg at day 30, 200 mg at day 50, 310 mg at day 75, 1700 mg at day 122 and 2100 mg at day 150.

Receptor assays

Concentrations of free receptors were determined in testicular homogenates (Goxe *et al.*, 1993). Briefly, 0.5–1 g of frozen testes (or pieces thereof) were reduced to powder at -80°C ,

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and then thawed in 1–3 ml of homogenization buffer (10 mmol Tris-HCl l^{-1} , pH 7.4, 3.8 mmol NaCl l^{-1} , 3 mmol KCl l^{-1} , 1 mmol phenyl-methyl sulfonyl fluoride (PMSF) l^{-1} , 1 mmol benzamidine l^{-1} and 0.04% (w/v) NaN_3) and homogenized at 4°C in a mortar with three strokes of a teflon pestle. Lumps were decanted and supernatant collected for receptor assay and protein measurement (BCA kit from Pierce, Roissy, based on the spectrometric measurement of the complexes between bicinchoninic acid and Cu^{2+} ions as reduced by peptide bonds).

Purified hCG (batch CR127, NIH, Bethesda, MD) was labelled with ^{125}I using Iodogen (Pierce) as catalyst (Genty *et al.*, 1987). The labelled hormone was purified on a Sephadex G-25 column (Pharmacia, St-Quentin-en-Yvelines) and the specific activity of the resulting tracer was 800–1500 Ci $mmol^{-1}$. ^{125}I -labelled human FSH (Christophe *et al.*, 1993) was a gift from P. Robert (Faculté de Pharmacie, Paris). Crude commercial preparations of hCG (Chorulon; Intervet, Angers) and pig FSH (a gift from Y. Combarrous, INRA, Tours) were used for the determination at nonspecific binding.

Binding was determined at three dilutions (equivalent to 4, 12 and 35 mg of tissue) in 100 μ l of homogenization buffer and compared with a standard binding curve made with similar dilutions of a known pig testis homogenate, and normalized per mg protein. Total binding was determined in the presence of 40–100 nmol of ^{125}I -labelled hormone l^{-1} , and nonspecific binding in the presence of a 500–1000-fold excess of unlabelled commercial hormone preparation. After 16 h at 20°C, the incubations were stopped by dilution with 1.6 ml of ice-cold 10 mmol Tris-HCl l^{-1} , pH 7.4 buffer and tubes were centrifuged at 3500 g at 4°C for 12 min. The supernatant was aspirated and the radioactivity of the pellet was determined using a LKB gamma counter (Pharmacia). The difference between total and nonspecific binding was taken as specific binding. Results were calculated as the means $\pm$ SEM of triplicate determinations.

RNA characterization

Total RNA was isolated according to Chomczynski and Sacchi (1987), as modified by Puissant and Houdebine (1990). About 100 mg of testis were homogenized in 1 ml of 4.2 mol guanidinium thiocyanate l^{-1} (GuSCN, Fluka, L'Isle d'Abeau) solution containing 25 mmol sodium citrate l^{-1} , 0.5% (w/v) *n*-lauryl-sarcosine and 0.1 mol β -mercaptoethanol l^{-1} . To this mixture, 0.1 vol of 2 mol sodium acetate l^{-1} , pH 4 was added and a first extraction with 1 vol phenol (water-saturated) and 0.2 vol chloroform (chloroform: iso-amyl alcohol, 49:1) was performed. The mixture was placed on ice for 15 min, centrifuged for 30 min at 3000 g and RNAs in the aqueous phase were precipitated overnight at $-20^{\circ}C$ by the addition of 1 vol isopropanol. The RNA pellet was recovered by centrifugation (30 min, 3000 g , 4°C), rinsed with 70% ethanol, and dissolved in sterile water. RNAs were precipitated by the addition of 4–5 vol of 4 mol LiCl l^{-1} at 4°C for 2 h. The RNA was pelleted as above and redissolved in a minimum volume of sterile water. A last extraction with 1 vol of chloroform-iso-amyl alcohol was performed. After 15 min on ice, the aqueous phase was recovered by centrifugation (3000 g , 20 min). RNA

was precipitated overnight with 0.1 vol of sodium acetate (final concentration of 0.2 mol l^{-1}) and 2.5 vol ethanol and pelleted (30 min, 10 000 g , 4°C). After washing with 70% ethanol, RNAs were dissolved and quantified by absorbance measurements at 260 nm and 280 nm (absorbance ratio about two) and checked by agarose gel electrophoresis. Aliquots of 20 and 40 μ g were stored in 70% ethanol, 0.2 mol sodium acetate l^{-1} at $-20^{\circ}C$.

Northern blot analysis was performed after electrophoresis on a 1.66% agarose gel in electrophoresis buffer (20 mmol NaH_2PO_4 l^{-1} , pH 7, 5 mmol sodium acetate l^{-1} , 1 mmol EDTA l^{-1} , 2.2 mol formaldehyde l^{-1}). Twenty μ g of each RNA was denatured (10 min, 65°C) in 10 μ l denaturation buffer (electrophoresis buffer supplemented with 45% (v/v) formamide, 15% (v/v) formaldehyde), cooled at 0°C, and supplemented with 10 μ l of 20% (w/v) Ficoll, 2 mmol EDTA l^{-1} , 0.2% (w/v) bromophenol blue. After migration, the gel was washed successively with sterile water and $10\times$ SSC (1.5 mol NaCl l^{-1} , 0.15 m sodium citrate l^{-1} , pH 7) and transferred overnight to Zeta Probe membrane (Biorad, Ivry-sur-Seine). After rinsing, filters were fixed for 2 min under UV irradiation.

Filters were then pre-hybridized for 3 h at 65°C in 0.5 mol sodium phosphate l^{-1} , pH 7.2, 7% (w/v) sodium dodecyl sulfate solution containing 5 g skimmed milk powder l^{-1} and 25 mmol EDTA l^{-1} . Filters were then hybridized overnight under the same conditions with the full-length cDNA probes for pig LH receptor (Loosfelt *et al.*, 1989) and pig FSH receptor (Remy *et al.*, 1995). Each probe was labelled by random priming with the Amersham Multiprime kit using [α - ^{32}P]dCTP (3000 Ci $mmol^{-1}$) for 3 h at room temperature to specific activities of approximately 10^9 c.p.m. μ g $^{-1}$. The filters were submitted to autoradiography (Kodak X-Omat S films, with intensifying screen) and band densities measured with the Pharmacia Image Master system and normalized to 1 mg total RNA. β -Actin probe (Alonso *et al.*, 1986) was not used for correction because its hybridization between days 30 and 75 was five times more intense than at day 20 and after day 120. Instead, ethidium bromide staining of ribosomal RNAs was used to check equal loading of electrophoresis tracks. Results were calculated as the means $\pm$ SEM of triplicate or quadruplicate determinations.

Results

Expression of LH and FSH receptors

The expression of LH and FSH receptors with postnatal age is displayed (Fig. 1). Receptors were detected with difficulty at day 10 (not shown). The amount of each receptor as c.p.m. tracer bound per mg protein was very low at day 20 and increased sharply at day 30 for the LH receptor (Fig. 1a) and at day 50 for the FSH receptor (Fig. 1b); noticeably, testis descent occurred around day 50. After day 75, binding concentrations declined slightly. When the total amount of receptors was estimated as pmol per testis, the increase was especially noticeable after day 75, which parallels the onset of testosterone production observed in previous studies (Boussit, 1989).

Fig. 1. Expression of gonadotrophin receptors in rabbit testes during postnatal development. Free receptors were measured in testis homogenates with ^{125}I -labelled (a) LH or (b) FSH. Specific binding was calculated as ($\circ$) c.p.m. mg protein $^{-1}$ or ($\blacksquare$) pmole per testis. Results were calculated as the means $\pm$ SEM of triplicate determinations.

Northern blots analysis

The age-dependent changes in the pattern of receptor RNAs encoding LH and FSH as detected by northern blots are shown (Fig. 2), and the absorbances of the main hybridization bands after densitometry are also given (Fig. 3). Adult (180 days) testis revealed four bands for the LH receptor probe (1.2, 2.5, 3 and 4.2 kb). However, the intensity of the 4.2 kb band was faint in some animals, independent of age; thus it was not used for quantification. Two bands at 1.2 and 2.2 kb were found for the FSH receptor (Fig. 2b). When earlier developmental stages were studied, the intensities of the low molecular mass bands (1.2 kb) normalized to 1 mg total RNAs were only slightly lower than in the adult (Fig. 3), except at day 20 for the FSH receptor. However, the high molecular mass bands of both receptors were significantly less intense than their adult

Fig. 2. Northern blots of RNAs encoding gonadotrophin receptors from rabbit testes during postnatal development. Total RNA was extracted from individual rabbit testes of the indicated age (days) and 20 μg were run on agarose gel and transferred before hybridization with (a) cDNA probe for the pig LH receptor (R-LH) or (b) cDNA probe for pig FSH receptor (R-FSH). For each figure: upper part, northern blot; lower part, ethidium bromide staining of ribosomal RNAs 28s and 18s (rib.). The figure is representative of one set of RNAs. Note that autoradiographic exposure was carried out for 10 days (LH receptor) and 3 days (FSH receptor).

Fig. 3. Changes in the relative intensities of the hybridization bands of the gonadotrophin receptors in rabbit testes with postnatal age. The intensities of the bands detected by northern blot in Fig. 2 were plotted versus postnatal age of the rabbits for (a) the LH receptor and (b) the FSH receptor. Absorbances were normalized per 1 mg total RNA. (■) 2.5 kb, (▲) 3 kb, (○) 1.2 kb, (●) 1.2 kb. Each sample was run in triplicate or quadruplicate and values shown are means $\pm$ SEM of arbitrary units.

counterparts: at days 20 and 30, the intensity of the LH receptor was 50% of that at day 180 (Fig. 3a), whereas intensity of the 2.2 kb band of the FSH receptor was only 20–25% of that at day 180 (Fig. 3b). These results indicate that the relative content in high molecular mass RNAs increased with the stage of development for both receptors, especially after 30 days of age, in parallel with the protein itself. The best correlation was found between the acquisition of the FSH receptor and the intensity of its 2.2 kb RNA. For the LH receptor, the increase in relative receptor amount occurred as early as day 30, while the amount of RNA hybridization was only slightly higher than at day 20.

Discussion

The expression of LH and FSH receptors in the testis has been correlated consistently with activity and/or development of their cognate tissues in various mammalian species. During fetal ontogenesis, expression of receptors occurs soon after sexual differentiation in pigs (Goxe *et al.*, 1993) and rats (Warren *et al.*, 1984; Rannikki *et al.*, 1995). Between stages VII–VIII and XIII–II of spermatogenesis, the concentration of RNAs encoding the FSH receptor in the rat seminiferous

epithelium underwent a threefold increase (Heckert and Griswold, 1991). The neonatal rabbit testis exhibits a slow-down of growth around day 20–24 followed by a wave of development (Allen, 1904). Thus, it was not surprising to find that during early (days 30–50) prepubertal development of the rabbit testis, the expression of both receptors per mg protein increased, and that their increase was accompanied by previously described physiological events: onset of spermatogenesis (days 45–60) and testosterone secretion (after day 60). Furthermore, the whole testis content in both receptors rose steadily, indicating a continuous development of both Leydig and Sertoli cells with gain in testicular mass. Such a correlation with testicular growth has already been reported in pigs (Peyrat *et al.*, 1981; Goxe *et al.*, 1993).

Previous work has demonstrated the existence of a number of transcripts (ranging from 1.2 kb up to 7.5 kb) in the gonads of all mammalian species studied for the LH receptor: pigs (Loosfelt *et al.*, 1989), rats (McFarland *et al.*, 1989), humans (Minegishi *et al.*, 1990), and mice (Gudermann *et al.*, 1992); and for the FSH receptor: rats (Sprengel *et al.*, 1990), humans (Minegishi *et al.*, 1991), sheep (Yarney *et al.*, 1993), monkeys (Gromoll *et al.*, 1993), horses (Robert *et al.*, 1994), and cattle (Houde *et al.*, 1994). Since the minimal expected coding length is 2.1 kb, those transcripts of shorter length are considered as noncoding or potentially encoding for truncated forms of the receptors, representing the extracellular, possibly soluble, domain of the receptor (Segaloff and Ascoli, 1993). Alternatively, truncated forms of the receptor may be attributed to proteolysis (Kellokumpu and Rajaniemi, 1985; West and Cooke, 1991) rather than to translation of short transcripts. In any case, soluble forms of the receptor would not be detected in the particulate fractions used in our study. The absence (or very low amount) of hormone binding was correlated with the presence of only a short transcript (1.2 kb) and with a low amount or absence of high molecular mass RNA. This situation is well illustrated by the FSH receptor, for which even the increase of the sole 1.2 kb RNA between days 20 and 30 is accompanied by a persistent absence of FSH binding. For the LH receptor, this holds true at day 20, while at day 30 the protein seems to be overexpressed as compared with the small amounts of 2.5 and 3 kb RNAs. Nevertheless, situations have already been described where the protein:RNA ratio of the LH receptors change as a result of physiological events such as lactation in rabbit does (Kermabon *et al.*, 1994) or subsequent to *in vitro* hormonal stimulation of cultured pig granulosa cells (Goxe *et al.*, 1992). In the latter system, mRNAs encoding the FSH receptor have also been reported to increase in the face of decreased hormone binding (Sites *et al.*, 1994).

The expression of the higher molecular mass transcripts of the rat LH receptor has been correlated with expression of the receptor itself, both at the onset of ovarian development (Sokka *et al.*, 1992) and upon maturation of testicular Leydig cells (Zhang *et al.*, 1994). Similar findings have been reported for the FSH receptor in rat gonads (Rannikki *et al.*, 1995). The origin of the variety of gonadotrophin receptor transcripts may be primarily attributed to heterogeneity in the length of their 3'-untranslated regions. Three sites of transcription termination, separated by about 2 kb from one another, have been found in the rat gene (Hu *et al.*, 1994). It may be that, in rabbits,

termination could take place at one proximal site for the LH and FSH receptors (2.5 and 2.2 kb bands, respectively) and at two additional distal sites for the LH receptor (3 and 4.2 kb bands). The potency of the 4.2 kb terminator could be lower than that of the 3 kb terminator, giving rise to some irregularities in the intensity of the corresponding band. In addition, transcript diversity may also derive from extensive alternative splicing of the pre-messenger RNAs (Aatsinki *et al.*, 1992; Bacich *et al.*, 1994) and from exon skipping (Koo *et al.*, 1994). Thus, 1.2 kb mRNAs, essentially devoid of the region coding for the transmembrane domain of the LH receptor, could arise from splicing between the 5' end of the tenth exon and 3' acceptor sites within the 11th exon (Loosfelt *et al.*, 1989; Bernard *et al.*, 1990; Koo *et al.*, 1994). Similar truncated mRNAs of the FSH receptor have also been reported (Gromoll *et al.*, 1992).

The low abundance of the larger transcripts in the early postnatal gonad may also be attributed to their reduced lifespan. This suggestion is in keeping with previous results indicating that the smaller transcripts would always be present in the gonads, even immature gonads (Aatsinki *et al.*, 1992; Rannikki *et al.*, 1995) or results obtained after downregulation induced by high doses of hCG (Wang *et al.*, 1991; Hsueh and LaPolt, 1992; Lakkakorpi *et al.*, 1993; Chuzel *et al.*, 1995). Indeed, hCG did not affect the rate of transcription of the LH receptor gene, but reduced the stability of its larger mRNAs (Lu *et al.*, 1993). The decrease in mRNA lifespan could be attributed to short-lived proteins whose translation is inhibited by cycloheximide (Goxe and Salesse, 1993; Shi and Segaloff, 1995).

Another molecular mechanism accounting for the presence of short transcripts may be premature termination of transcription. Indeed, one hypothesis concerning transcription termination of polyintrinsic genes suggests that, beyond the last exon, sequences typical of 3'-untranslated region, such as polyadenylation sequences (AAUAAA or similar), would be recognized by the polymerase II complex (Proudfoot, 1989; Manley, 1995). Such sequences are found in introns 3, 4 and 10 of the rat LH receptor genes, where transcripts are stopped after the 3rd, 4th and 10th exons (Koo *et al.*, 1994). This could also be the case in the immature rabbit testis before day 30. Subsequently, the transcriptional and post-transcriptional machinery of the testis would reach a functional level allowing completion of transcription, correct splicing and stabilization of RNAs, although still leaving significant amounts of truncated transcripts. The rabbit testis thus appears to provide a suitable model for study of regulatory mechanisms (possibly hormonal) involved in the generation of translatable mRNAs of the gonadotrophin receptors since, unlike in the testes of other mammalian species, only a limited number of bands were identified by northern blotting, thus offering an advantage for the study of the generation, lifespan and role of the short transcripts.

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