A switch from continuous to episodic testicular testosterone release in response to pulsatile LH stimulation in juvenile rhesus monkeys (*Macaca mulatta*)

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Abstract

This study examined the ontogeny of the testicular testosterone response to precocious pulsatile LH stimulation in the juvenile rhesus monkey. LH stimulation was achieved with an i.v. infusion (one pulse every 3 h) of either single-chain human (sch)LH, administered alone or in combination with recombinant human (rh)FSH, or recombinant monkey (rm)LH in combination with rmFSH. Homologous gonadotropin treatment resulted in an adult profile of circulating mLH concentrations. The schLH infusions produced a similar pulsatile pattern in circulating LH with peak concentrations of approximately 5 IU/l. Although a robust testicular testosterone response was observed after 24 h of intermittent LH stimulation, surprisingly testosterone release at this time was continuous.

The apulsatile mode of testosterone secretion, however, did not persist, and a switch to an unequivocal episodic mode of secretion, comparable to that observed in adult monkeys, occurred by day 4 of LH stimulation. FSH did not influence the pattern of the testosterone response. We conclude from these findings that progenitor Leydig cells in the primate testis are able to respond rapidly to a physiological LH stimulus. While the cell biology underlying the switch from a continuous to a pulsatile mode of testosterone secretion remains unclear, we suggest that this phenomenon may be related to the hypothesis that episodic testosterone secretion is required for the operation of the neuroendocrine axis governing testicular function.

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Introduction

Postnatal production of testosterone by the Leydig cell is regulated by the pituitary secretion of luteinizing hormone (LH; Ewing & Keeney 1993, Saez 1994, Benton *et al.* 1995, Huhtaniemi 1996, Lejeune *et al.* 1998, Mendis-Handagama & Ariyaratne 2001) acting via the transmembrane LH receptor (LHR) that is coupled to the G-protein-cyclase-cyclic AMP dependent protein kinase A signal transduction pathway (Ascoli *et al.* 2002). In adulthood, the pattern of testosterone secretion is typically episodic and temporally coupled to the intermittent discharge of LH from the pituitary (Plant 1981, Ewing & Keeney 1993, Ramaswamy *et al.* 1998, 2000a).

In higher primates, the characteristic pulsatile mode of testicular testosterone secretion in the adult is preceded by a protracted phase of quiescence in the hypothalamic–pituitary–gonadal (HPG) axis that is initiated in late infancy and maintained until puberty (Plant 1994). During the hypogonadotropic juvenile phase, however, an adult pattern of testosterone secretion may be elicited by prematurely activating a pubertal pattern of endogenous

gonadotropin release (Plant *et al.* 1989). While the precise dynamics of testicular testosterone secretion at the onset of puberty has not been described in either man or monkey, it is reasonable to presume that, since LH secretion is pulsatile at this stage of development (Wu *et al.* 1993, Manasco *et al.* 1995, Albertsson-Wikland *et al.* 1997, Suter *et al.* 1998, Mitamura *et al.* 1999, Veldhuis *et al.* 2001, Crofton *et al.* 2004), the release of testicular testosterone would also be episodic.

We were therefore surprised to observe continuous release of testosterone, in response to initial intermittent stimulation of the juvenile rhesus monkey testes with exogenous LH, in two studies requiring premature activation of testicular function. This serendipitous finding is presented here.

Materials and Methods

Animals

Fourteen juvenile male rhesus monkeys (*Macaca mulatta*, 14–19 months old, 2.0–3.2 kg body weight) were used.

Eight of these animals comprised a previously published study aimed at establishing the relative role of LH and follicle-stimulating hormone (FSH) in Sertoli cell proliferation at the time of puberty (Ramaswamy *et al.* 2000b). Four other animals, which served as a control group in an unpublished study, had been implanted with empty s.c. Silastic capsules at least 6 weeks before initiation of LH stimulation. Two additional animals were used to define specifically the initial development of the testicular testosterone response to LH stimulation. Here, it should be noted that, in the rhesus monkey, the pubertal initiation of nocturnal testosterone secretion occurs at approximately 30–36 months of age (Plant 1985).

Access to venous circulation

Each animal was surgically implanted with two indwelling catheters, which provided continuous access to the venous circulation, using a sterile technique under sodium pentobarbital anesthesia (25 mg/kg body weight, i.v. Nembutal Sodium Solution, Abbott Laboratories, Chicago, IL, USA) or isoflurane inhalation (1–2.5% with oxygen), as described previously (Ramaswamy *et al.* 2003, Shahab *et al.* 2003). The post-surgical care of the first group of eight animals has been described previously (Ramaswamy *et al.* 1999, 2000b), and this was also employed for the two animals used to study the early testicular response to LH stimulation. The remaining four animals each received a single i.m. injection of penicillin (Pen-G, 40 000 U/kg body weight, Phoenix Pharma Inc., MO, USA) on the day of surgery, and twice-daily i.v. injections of a broad-spectrum antibiotic cefazolin sodium (25 mg/kg body weight, Kefzol, Apotekon, Princeton, NJ, USA or Ancef, GlaxoSmithKline) and an analgesic (Ketofen, 2 mg/kg body weight, Fort Dodge Animal Health, IA, USA) for 4 days. The routine care of animals fitted with a jacket and tether system, and housed in specialized remote-sampling cages has been described previously (Ramaswamy *et al.* 1999, 2003). The animals were maintained under a controlled photoperiod (lights on 0700 to 1900 h) in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals; the experimental procedures were approved by the Institutional Animal Care and Use Committee.

Hormone infusates

Stock solutions of recombinant single-chain human (sch)LH (300 IU/ml), recombinant human (rh)FSH (200 IU/ml) and recombinant monkey (rm)FSH (National Hormone and Peptide Program (NHPP, rec moFSH-I-1, AFP6940A) and rmLH (NHPP, rec moLH-I-1, Bio, AFP6936A), both at 1 µg/10 µl, were prepared in Dulbecco's PBS (DPBS without CaCl₂ and MgSO₄, Gibco).

Experimental design

Stimulation with schLH The details of this experiment, including the preparation of custom gonadotropin infusates for each animal, have been reported previously (Ramaswamy *et al.* 2000b). In brief, groups of four juvenile monkeys each received, beginning on day 0, intermittent i.v. infusions of schLH, either alone or in combination with rhFSH (3 and 2 IU/kg per pulse respectively), as a 1-min bolus pulse (2 ml) every 3 h for 11 days. A single blood sample was taken prior to initiation of the hormone infusion on day 0. The profiles of circulating gonadotropin concentrations resulting from the intermittent infusion of the recombinant hormones, and the testicular testosterone responses to exogenous stimulation with LH, were determined in a series of frequent blood samples. Samples were collected, during a 3-h inter-pulse interval, at ~5–10 min before and at 3, 20, 40, 60 and 170 min after a pulse infusion of schLH on days 1 and 8 of the experiment. In two of the four animals receiving schLH alone, additional blood samples were collected at 80 and 120 min after the pulse infusion.

Two additional animals received an intermittent infusion of schLH alone, at the dose described above. In this experiment, blood samples were collected at ~5–10 min before and at 3, 20, 40, 80 and 170 min after a pulse infusion of vehicle (DPBS), and after the first, second and fourth pulse during the initiation of the schLH intermittent infusion.

Stimulation with rmLH A custom infusate of rmLH (in combination with rmFSH) was prepared for each animal in sterile DPBS containing 2 mg/ml cefazolin sodium (Kefzol or Ancef) and 1% serum from the respective monkey. The doses of rmLH and rmFSH used were 600 and 30 ng/kg per pulse respectively. These doses for rmLH and rmFSH were selected since they produced circulating levels of the respective hormone in the range seen in the adult monkey (Ramaswamy *et al.* 1998, El Majdoubi *et al.* 2000).

For this experiment, the animals each received, beginning on day 0, intermittent i.v. infusions of rmLH (in combination with rmFSH) as a 1-min bolus pulse (1 ml) every 3 h. Circulating concentrations of infused rmLH, and the testicular testosterone response were determined in a series of frequent blood samples. Samples were collected, during a 3-h inter-pulse interval, at ~5–10 min before and at 5, 20, 40, 60, 80, 100 and 170 min after infusion of a gonadotropin pulse on days 1, 2, 4 and 8–10 of the experiment. In order to obtain baseline hormone concentrations, an identical series of blood samples was collected during a 'mock' 3-h window a few days prior to the initiation of gonadotropin treatment.

Assays

Gonadotropins Circulating concentrations of infused schLH were determined using an assay kit specific for hLH

(Technicon Immuno-1 System, Bayer) as described previously (Ramaswamy *et al.* 2000b). Circulating concentrations of infused rmFSH and rLH were determined using homologous (cynomolgus) RIAs as described previously (Ramaswamy *et al.* 1998, El Majdoubi *et al.* 2000). The mean sensitivity of the LH assay was 0.12 ng/ml and the intra- and inter-assay coefficients of variation were less than 5% and 4.4% respectively.

Testosterone Circulating concentrations of testosterone were determined using either a previously described RIA employing antiserum T3-125 (Plant 1981) or a commercially available solid-phase RIA kit (Coat-A-Count, Total T (TKTT), Diagnostic Products Corporation, LA, USA). The mean sensitivity of the two assays was approximately 0.065 and 0.028 ng/ml respectively. The intra- and inter-assay coefficients of variation for the former assay were 7.7% and 12.9% respectively; those representative of the latter assay were 4.5% and 7.9% respectively.

Statistical analyses

The significance of differences between mean hormone concentrations was determined by Student's *t*-test, or by one-way or multifactor ANOVA with repeated measures followed by the Student-Newman-Keuls multiple-range test, as appropriate. Hormone levels below the sensitivity of the assay were assigned a value equivalent to the minimum detectable concentration in the respective assay. All data are expressed as means $\pm$ S.E.M. and statistical significance was accepted at $P \leq 0.05$.

Results

Stimulation with schLH

Before stimulation with schLH, circulating concentrations of immunoactive hLH were undetectable (Ramaswamy *et al.* 2000b). Initiation of the intermittent schLH infusions produced an episodic pattern in the circulating concentration of immunoactive hLH with peak values on days 1 and 8 ranging from 3.60 to 6.20 IU/l and from 2.20 to 4.30 IU/l respectively (Fig. 1). The mean concentrations of immunoactive hLH in the circulation during a 3-h window of frequent blood sampling on days 1 and 8 of LH stimulation, alone, were 1.86 ± 0.16 and 1.24 ± 0.18 IU/l respectively (Table 1). The corresponding plasma LH concentrations in the group treated in combination with rhFSH were similar (Fig. 1 and Table 1). In both groups, LH concentrations on days 1 and 8 were significantly different.

The baseline testosterone concentration in the circulation prior to initiation of the schLH infusion was 0.39 ± 0.09 ng/ml (Ramaswamy *et al.* 2003), and by day 1 of stimulation, a striking 10-fold increase in testosterone

Figure 1 Circulating concentrations (means $\pm$ S.E.M.) of immunoactive hLH (○) and testosterone (●) during selected 3-h inter-gonadotropin pulse intervals following the initiation, on day 0, of an intermittent i.v. infusion of schLH, either alone (top panels) or in combination with rhFSH (bottom panels). Note the switch from an initial continuous mode of testosterone release on day 1 (D1) to that of pulsatile mode of release of this steroid on day 8 (D8); the latter is reminiscent of the pattern of testosterone release in the adult monkey (Plant 1981). a, significantly different from all other time points.

concentration was observed (Fig. 1). At this time, there was no evidence of an episodic pattern of testosterone secretion (Fig. 1). By day 8 of schLH stimulation, however, the pattern of testosterone secretion had switched to a distinct episodic mode, as reflected by the time course of circulating testosterone (Fig. 1). The mean plasma testosterone concentrations during the initial pulsatile phase of secretion (day 1) and the subsequent pulsatile mode of release (day 8) of this steroid were 4.04 ± 0.54 and 2.96 ± 0.63 ng/ml respectively (Table 1). The more comprehensive sampling paradigm employed in two of the four animals receiving schLH, alone, substantiated the pulsatile mode of testosterone release during the initial day of LH stimulation (data not shown).

In the two additional animals, in which the initial testicular testosterone response to LH stimulation was monitored, the circulating profiles of immunoactive hLH were similar to those seen in the first experiment (Fig. 2). As in the first group of animals, a robust testosterone response was also observed after 24 h of LH stimulation. Circulating testosterone levels during the first, second and fourth schLH pulse, however, remained within the range observed during vehicle infusion (Fig. 2).

The addition of rhFSH to the LH infusion did not influence the overall testicular testosterone response to intermittent stimulation with schLH (Fig. 1 and Table 1). During combined gonadotropin treatment, the mean testosterone concentrations in the circulation on days 1 and 8 were 2.32 ± 0.27 and 2.24 ± 0.33 ng/ml respectively (Table 1).

Table 1 Circulating concentrations (means ± S.E.M.) of immunoactive LH and testosterone during selected 3-h windows of frequent blood sampling in juvenile monkeys (*n*=4 per group) treated with pulsatile i.v. infusion of heterologous or homologous preparations of gonadotropins. ^a, significantly different from day 1; ^b, significantly different from days 4 and 8–10.

	schLH (alone)		schLH (with rhFSH)		rmLH (with rmFSH)	
	LH (IU/l)	Testosterone (ng/ml)	LH (IU/l)	Testosterone (ng/ml)	LH (ng/ml)	Testosterone (ng/ml)
PRE					0.11 ± 0.01	0.15 ± 0.07
Day 1	1.86 ± 0.16	4.04 ± 0.54	2.21 ± 0.64	2.32 ± 0.27	0.92 ± 0.09	4.45 ± 0.08
Day 2					1.14 ± 0.27	6.01 ± 1.37 ^b
Day 4					0.85 ± 0.08	3.21 ± 0.46
Days 8–10	1.24 ± 0.18 ^a	2.96 ± 0.63	1.61 ± 0.03 ^a	2.24 ± 0.33	0.90 ± 0.06	2.35 ± 0.20

Stimulation with rmLH

Prior to treatment with homologous gonadotropins, circulating concentrations of endogenous LH were undetectable (Table 1). Following the initiation of the intermittent infusion of rmLH (in combination with rmFSH), the expected episodic pattern of circulating LH concentrations was noted (Fig. 3). Mean LH concentration on days 1, 2, 4 and 8–10 were similar (Table 1).

The mean baseline plasma testosterone concentration before the initiation of the rmLH infusion was 0.15 ± 0.07 ng/ml. Stimulation of the testes with homologous gonadotropin resulted in a marked increase in the circulating concentration of testosterone, which by day 1 had achieved a mean value of approximately 4.5 ng/ml (Fig. 3). Similar to the situation noted with the schLH infusion, there was no evidence for episodic testosterone release at this time (Fig. 3). An episodic discharge of this steroid, however, was noted as early as day 2 of intermittent rmLH treatment (Fig. 3) and, by day 4, the pattern of testosterone secretion was unequivocally episodic; a mode of release that was sustained for the 8–10 days of treatment (Fig. 3).

The switch from a continuous to pulsatile mode of testosterone release was associated with a progressive decrease in mean testosterone concentrations, with values of 6.01 ± 1.37, 3.21 ± 0.46 and 2.35 ± 0.20 ng/ml noted on days 2, 4 and 8–10 respectively. The mean testosterone concentration on day 2 was significantly different from those on subsequent days (Table 1).

Discussion

Episodic secretion in the hypothalamic gonadotropin-releasing hormone (GnRH)–pituitary LH–testicular testosterone axis is a hallmark of this neuroendocrine system in adult primates and other mammals (Plant 1994). It is therefore of interest that precocious intermittent stimulation of the juvenile monkey testes with either homologous or heterologous LH, which mimics an ‘adult’ drive to the interstitial cells, should initially produce an apulsatile testicular testosterone response. A similar continuous mode of testosterone release in response to pulsatile LH

Figure 2 Circulating concentrations of immunoactive hLH (○) and testosterone (●) in two additional juvenile monkeys during infusion of vehicle (left-hand panels) and during first, second and fourth inter-gonadotropin pulse intervals immediately following initiation of an intermittent i.v. infusion with schLH alone. Note the absence of a testicular testosterone response during this period of LH stimulation.

stimulation has also been described in adult rams rendered hypogonadotropic by immunization against GnRH (Chase *et al.* 1988). In the present study, circulating testosterone concentrations prior to the initiation of pulsatile LH stimulation were reminiscent of those in castrated adults (El Majdoubi *et al.* 2000), where they remained during the first four pulses of LH stimulation. Interestingly, a marked testosterone response by the juvenile testes required between five and nine pulses of LH stimulation; and by 24 h of intermittent LH stimulation, the levels of this steroid had reached concentrations in the range of those typically observed in adult monkeys (Plant 1981, Ramaswamy *et al.* 1998). Therefore, it is reasonable to conclude that within 12–24 h of initiating gonadotropin stimulation of the juvenile monkey testis, the biosynthetic machinery for LH signaling and steroidogenesis is recruited in the interstitial cells that are destined to become adult Leydig cells.

The mechanisms responsible for the initial continuous pattern of testosterone release remain to be determined. Several possibilities may be considered. First, during the 24 h of the intermittent LH infusion, the progenitor Leydig cells in the interstitium of the juvenile testis may not receive a pulsatile LH signal from the vascular compartment. This view is supported from observations in rats that LH or hCG stimulation results in changes in interstitial blood flow and capillary permeability (Setchell & Sharpe 1981, Turner & Rhoades 1995, Setchell *et al.* 2002). Secondly, it has been suggested that the endothelial cells of the testis may play a role in mediating LH-stimulated testosterone secretion (Ghinea & Milgrom 1995, Setchell *et al.* 2002), and if this is the case, this relationship may change with time. Thirdly, the release of testosterone from the progenitor Leydig cells may initially be intrinsically continuous. In this regard, it is well established that, at the time of puberty, expansion of this population of cells is associated with maturation of cellular, biochemical and molecular machinery necessary for the synthesis and secretion of testosterone (Chemes *et al.* 1985, Saez 1994, Benton *et al.* 1995, Rey *et al.* 1995, Ge & Hardy 1998, Lejuene *et al.* 1998, Mendis-Handagama 2000, Mendis-Handagama & Ariyaratne 2001, Prince 2001, O'Shaughnessy *et al.* 2002, Haider 2004). Fourthly, during the first 24 h of LH stimulation, secretory activity by progenitor Leydig cells may not be synchronized. This view is based on the observations that during pubertal testicular development recruitment of progenitor Leydig

Figure 3 Time course of changes in circulating concentrations (means $\pm$ S.E.M.) of monkey LH (○) and testosterone (●) before (PRE) and on days 1, 2, 4 and 8–10 after the initiation of an intermittent i.v. infusion with rmLH (in combination with rmFSH). Note the gradual switch between days 2 and days 8–10 from an apulsatile to a pulsatile mode of testosterone release in association with a decrease in the mean concentration of testosterone in the face of an invariant LH stimulation. *a*, significantly different from all other time points.

cells occurs following exposure to gonadotropins (Mancini *et al.* 1963, Fouquet *et al.* 1984, Prince 1984, 2001, Nistal *et al.* 1986, Saez 1994). Such a developmental event involving differentiation of more than one cell type (i.e. fibroblasts, and mesenchymal and immature Leydig cells) may result in subpopulations of steroidogenic cells that respond to LH stimulation with variable latencies. Lastly, but unlikely in our view, a pulsatile mode of testosterone release from interstitial cells may not be initially transmitted to the vascular compartment.

Whatever the explanation, a pulsatile profile in the release of testosterone had emerged by day 2 of LH stimulation, although a component of the release pattern remained continuous, as reflected by elevated basal testosterone levels. The pulsatile mode of testosterone secretion continued to mature while the continuous pattern of testosterone release regressed and, by day 8, the profile of circulating testosterone was similar to that observed in normal adult monkeys (Plant 1981). It is to be noted, however, that the switch from a continuous to a fully adult pulsatile mode of testosterone release was generally associated with a reduction in testicular testosterone secretion as reflected by a decline in mean levels of this steroid in the circulation.

At the cellular level, the mechanisms involved in the maturation of progenitor Leydig cells in to adult Leydig cells may include, in addition to those resulting directly from activation of LH signaling, autocrine actions of testosterone on the Leydig cell and/or paracrine actions of Sertoli cell factors induced secondarily in response to androgen receptor activation (Hardy *et al.* 1990, Gnani *et al.* 1997, Hales 2002, Haider 2004). Additionally, it is to be recalled that the testes are innervated by both intrinsic and extrinsic systems, which are known to exhibit a marked degree of plasticity during the peripubertal period (Prince 1992, Setchell *et al.* 1994, Mayerhofer 1996, Frungieri *et al.* 2000, Suburo *et al.* 2002, Geigerseder *et al.* 2004). Thus, it may also be possible that neuronal remodeling resulting from the exogenous LH stimulation of the juvenile testes may contribute to the switch from a continuous to a pulsatile mode of testosterone release.

Whether the initiation of testicular testosterone secretion during spontaneous puberty involves an initial continuous phase of steroid release is unclear. In this regard, it should be noted that during normal pubertal development the progenitor Leydig cells are probably not exposed to an abrupt, invariant and robust LH stimulation, as was produced in the present study. Instead, both LH pulse frequency and amplitude increase gradually (Plant 1994, 2001), which, in turn, would provide a progressively ascending LH stimulus to the progenitor Leydig cells.

Finally, the finding that progenitor Leydig cells exhibit the potential to robustly secrete testosterone in a continuous mode in response to pulsatile LH stimulation raises the theoretical question of why adult Leydig cells respond to the same LH stimulus with an episodic pattern of

testosterone secretion. We speculate that this may be related to the notion that episodic testosterone secretion is required for the operation of the HPG axis governing the testes. Specifically, while functions of the seminiferous tubules and the accessory sex structures are maintained in the face of continuous exposure to testosterone, we suggest that the activity of the hypothalamic–pituitary component of this axis may be down-regulated by physiological levels of testosterone presented in a continuous fashion.

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