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Testicular Secretion of Inhibin in the Male Golden Hamster (*Mesocricetus auratus)*

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ABSTRACT: To identify the cellular source of inhibin in the male golden hamster, we have used complementary approaches, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Strong positive staining of the inhibin α subunit was observed in both the Sertoli and Leydig cells of the testes. No specific staining was observed for the inhibin βA subunit, whereas specific staining for the inhibin βB subunit was strongly positive in the Leydig cells. Inhibin pro-αC and inhibin B were detected in peripheral plasma, and testicular homogenate also contained large amounts of inhibin pro-αC and inhibin B. However, inhibin A was not detected either in peripheral plasma or in testicular homogenate. Plasma concentrations of inhibin pro-αC and inhibin B were significantly ($P < .001$) decreased 24 hours after orchidectomy. These results strongly suggest that the Leydig cells are the main source of dimeric inhibin B in the male golden hamster.

Key words: Inhibin B, inhibin pro-αC, Sertoli cells, Leydig cells, testis.

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Inhibin is a dimeric glycoprotein consisting of an α and 1 of 2 β subunits. Two related forms of the β subunit, βA and βB subunits, are expressed in the gonad, and 2 forms of dimeric inhibins (inhibin A and inhibin B) are secreted into the circulation (Ling et al, 1985; Miyamoto et al, 1985; Rivier et al, 1985; Robertson et al, 1985). On the other hand, the dimers of β subunits are termed *activins*, activin A (βA-βA), activin B (βB-βB), and activin AB (βA-βB), which stimulate secretion of follicle-stimulating hormone (FSH) and suppress androgen production (Vale et al, 1988). Inhibins and activins have also been shown to exert paracrine effects, autocrine effects, or both, within the gonads (Lin et al, 1989; Chen, 1993).

In the female hamster, both inhibin A and inhibin B can be detected in peripheral plasma, and their plasma concentrations increased at the time of new follicular development on the morning of estrus (Ohshima et al, 1999). The rise in inhibin A and inhibin B at this time is likely to be a consequence of the secondary FSH surge. In contrast, inhibin B is the major form of circulating dimeric inhibins in the male monkey (Foppiani et al, 1999; Ramaswamy et al, 2000) and in men (Illingworth et al, 1996). In the adult rat testis, mainly the Sertoli cells and to a lesser extent, the Leydig cells, secrete inhibin (Maddocks and Sharpe, 1989; Sharpe et al, 1999). It is also demonstrated that inhibin B is the major form of inhibin in the male rat (Sharpe et al, 1999). Our recent study has demonstrated that the testes secrete dimeric inhibins into the circulation, and this hormone is an important factor in the regulation of FSH secretion in the adult male golden hamster (Kishi et al, 2000). It was also reported that in experiments using different photoperiods, changes in plasma inhibin levels were associated with changes in testicular function in the male golden hamster (John et al, 1993). These results suggest that the circulating levels of inhibin may reflect testicular function in the male hamster. However, the cellular source and molecular forms of inhibin have not yet been clarified in the male golden hamster. In the present study, we examined the specific cellular localization of the 3 inhibin subunits (α, βA, and βB) in the testis using immunohistochemical approach, and the circulating forms of inhibin using enzyme-linked immunosorbent assay (ELISA) in the adult male golden hamster.

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Material and Methods

Animals

Adult male golden hamsters (*Mesocricetus auratus*) (3–5 months old) were used in the present study. They were housed in groups of 6–8 animals per cage in a room with controlled conditions of temperature, humidity, and lighting (14 hours of light, 10 hours of dark; lights on at 0500 hours. Food and water were available ad libitum.

Plasma and Tissue Samples

Trunk blood was collected at 1100 hours from a group of animals after decapitation. Another group of animals underwent orchidectomy at 1100 hours under ether anesthesia. The orchidectomized animals were killed by decapitation 24 hours after the operation and trunk blood had been collected. All blood samples were collected into heparinized centrifuge tubes, stored in ice, and centrifuged at 1700 × *g* for 15 minutes at 4°C immediately after completing the experiment. Plasma was separated and stored at −20°C until assayed for inhibin A, inhibin B, and inhibin pro-αC. Testes were also collected, homogenized (TOMY; SEIKO Company LTD, Tokyo, Japan) in 1 mL 0.85% NaCl (w/v), followed by centrifugation at 20 800 × *g* for 30 minutes at 4°C. Supernatant was separated and stored at −20°C until assayed for inhibins.

ELISA

Concentrations of inhibin A, inhibin B, and inhibin pro-αC in plasma and testicular homogenates were measured with an ELISA kit (Serotec Ltd, Oxford, United Kingdom). The characteristics of these inhibin dimer-specific assays have been previously described for female hamsters (Ohshima et al, 1999).

Immunohistochemistry for Inhibin α, βA, and βB subunits and 3β-Hydroxysteroid Dehydrogenase

The testicular tissue samples were immediately fixed in 4% paraformaldehyde (Sigma Chemical Company, St Louis, Mo) in 0.05 M PBS pH 7.4, and embedded in paraffin. The paraffin-embedded testes were serially sectioned at 6 μm thickness and placed on poly-L-lysine coated slide glasses (Dako Japan Company, Kyoto, Japan).

After tissue sections were deparaffinized with xylene, they were subjected to antigen retrieval by autoclaving in 0.01 M sodium citrate buffer (pH 6.0) at 121°C for 15 minutes. The sections were then incubated in 3% H_2O_2 in methanol at room temperature for 30 minutes followed by 0.5% casein-Tris saline (CTS; 0.05 M Tris-HCl with 0.15 M NaCl pH 7.6) at 37°C for 1 hour to quench nonspecific staining. Following this, the tissue sections were incubated for 16 hours at 37°C with polyclonal antibodies made against inhibin subunits and also steroid enzyme, at dilutions from 1:8000 to 1:16 000 in CTS. Antibody against each inhibin subunit was anti-[Tyr30]-porcine inhibin α chain (1-30)-NH2 conjugated to rabbit serum albumin ([Tyr30]-porcine inhibin α chain (1-30)-NH2 was kindly provided by Dr N. Ling, Neuroendocrine Inc, San Diego, Calif), anti-cyclic inhibin βA (81-113)-NH2 (#305-24-D), and anticyclic inhibin βB (80-112)-NH2 (#305-25-D), kindly provided by Dr W. Vale (The Salk Institute for Biological Studies, La Jolla, Calif). The 3β-hydroxysteroid dehydrogenase (3β-HSD) antibody used in the experiment was polyclonal antibody against human placental 3β-HSD raised in rabbits (kindly provided by Dr J.I. Mason, Green Center for Reproductive Science, University of Texas, Southern Medical Center, Dallas, Tex). After incubation with specific antibodies, the sections were treated with 0.25% (v/v) biotinylated goat anti-rabbit secondary antibody (Elite ABC kit; Vector Laboratories, Burlingame, Calif) in CTS for 1 hour at 37°C, and were subsequently incubated with 2% (v/v) avidin-biotin complex (Elite ABC kit) in CTS for 30 minutes at 37°C. The reaction products were visualized by treating with 0.025% 3.3′-diaminobenzidine tetrachloride (DAB, Sigma) in 10 mM Tris-buffered saline containing 0.01% H_2O_2 for 1–30 minutes.

Statistics

In the inhibin assays, dilutions of plasma and testicular homogenate were used to check for parallelism with the human inhibin standard by 2 × 2 point analysis. Plasma concentrations of inhibin B and inhibin pro-αC between precastration and postcastration were compared using Student's *t* test. Differences were considered to be significant when $P < .001$.

Results

Immunohistochemistry

Sections stained with anti-inhibin α subunit showed strong positive reaction for the inhibin α subunit within the seminiferous tubules, presumably the cytosol of the Sertoli cells (Figure 1b). There was also strong positive staining in the Leydig cells (Figure 1b). There was no immuno reaction for the inhibin βA subunit (data not shown). On the other hand, the inhibin βB subunit was positively stained in the Leydig cells (Figure 1c). The interstitial cells that were positively stained for inhibin subunits were also stained for 3β-HSD (Figure 1d). Localization of 3β-HSD was not observed in the Sertoli cells and germs cells. Normal rabbit serum did not show any immunostaining reactions either within the seminiferous tubules or interstitial tissues. (Figure 1a).

Characterization of Inhibin Pro-αC, Inhibin A, and Inhibin B Assay

Dose-dependent curves of serially diluted peripheral plasma and testicular homogenate showed parallelism to the standard curves produced with inhibin pro-αC and inhibin B (Figure 2a and c). Whereas plasma and testicular homogenate failed to show parallelism to the standard curve produced with inhibin A (Figure 2b).

Effect of Bilateral Orchidectomy on Plasma Levels of Inhibin Pro-αC and Inhibin B

Following orchidectomy, the plasma concentrations of inhibin pro-αC fell below the limit of assay detection (Figure 3a). The plasma concentrations of inhibin B (Figure 3b) also fell significantly ($P < .001$) 24 hours after orchidectomy.

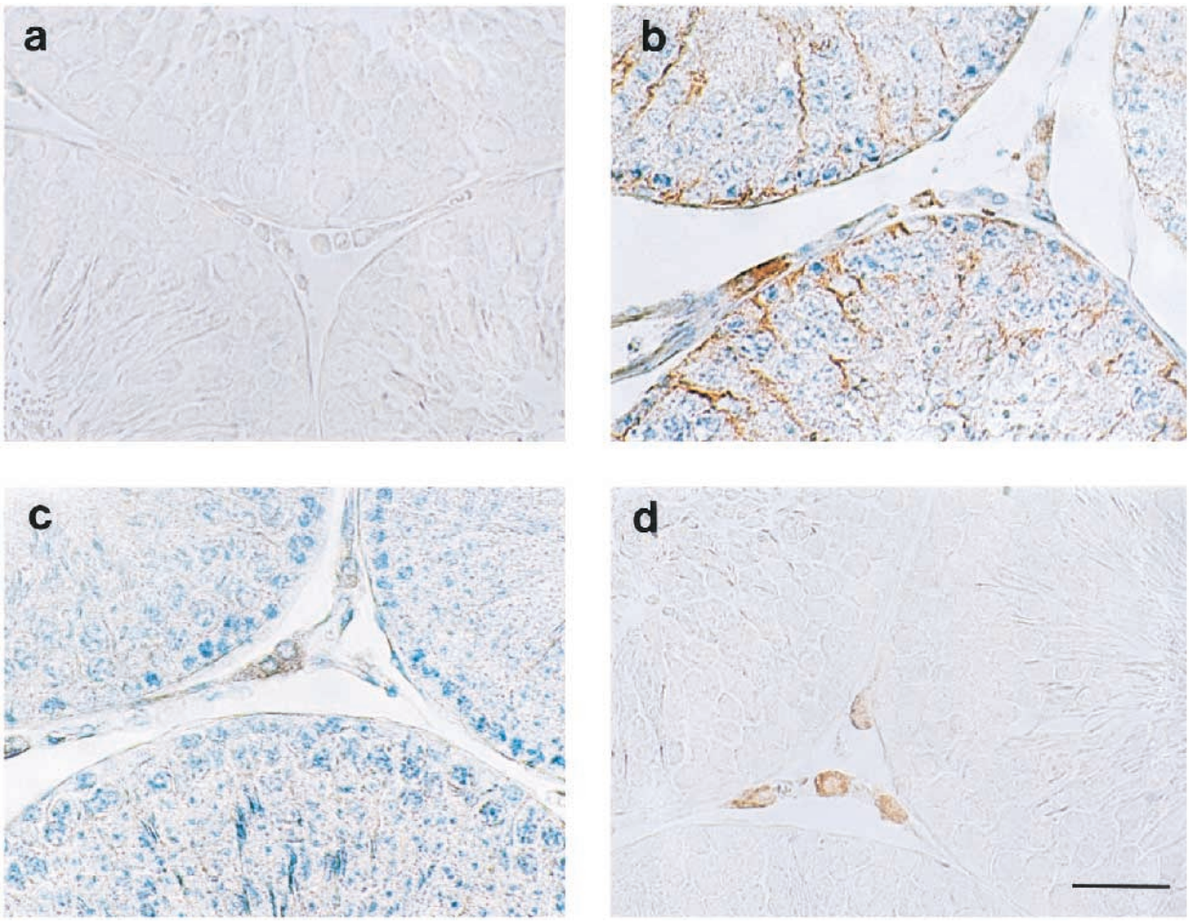

Figure 1. Immunohistochemical staining of inhibin subunits in hamster testes. **a**) Stained with normal rabbit serum; **b, c,** and **d,** stained with anti-inhibin α subunit, anti-inhibin βB subunit, and antiserum 3β-HSD, respectively. Bar = 100μm

Discussion

The present study demonstrated the immunolocalization of inhibin α and βB subunits in the Leydig cells of the testes in adult male golden hamsters. Immunohistochemical results also demonstrated colocalization of inhibin α and βB subunits with 3β-HSD in the same Leydig cell population of the testes, indicating that testicular Leydig cells have the ability to secrete testosterone and inhibin B in this species. There is a species-specific difference of the cellular source of inhibin in mammalian testes. The production of inhibin by Leydig cells is controversial. Anderson et al (1998) demonstrated that the inhibin α and βB subunits were localized in both the Sertoli cells and Leydig cells in humans. Nagata et al (1998) reported the immunolocalization of inhibin α, βA, and βB subunits in the Leydig cells and Sertoli cells of the testis in stallion. In neonatal and immature rats, immnolocalization of inhibin subunits or expression of inhibin messenger RNAs (mRNAs) were also observed in both the Leydig cells and Sertoli cells (Roberts et al, 1989; Shaha et al, 1989). Similarly, ovine fetal Leydig cells also express all 3 inhibin subunits at 40–90 days of gestation (Jarred et al, 1999). On the other hand, Sharpe et al (1999) reported that inhibin B is largely produced by Sertoli cells of the testis in the rat. However, in the present study, immunostaining for the inhibin βA subunit was not observed in the testes, indicating that inhibin A is not a major form of inhibin in adult golden hamsters. The present results are further supported by findings of Roberts et al (1989), who demonstrated that only the inhibin α subunit but not the inhibin βA, βB subunit mRNA signals were detectable in the Leydig cells of adult rat testes. Also, there are other reports that showed that inhibin βA, βB subunit mRNA signals were weaker in the Sertoli cells of the testes in the adult than in young rats. In the rat, only inhibin α and βB subunit mRNA signals were detectable by S1 nuclease analysis (Meunier et al, 1988). In the present study, the

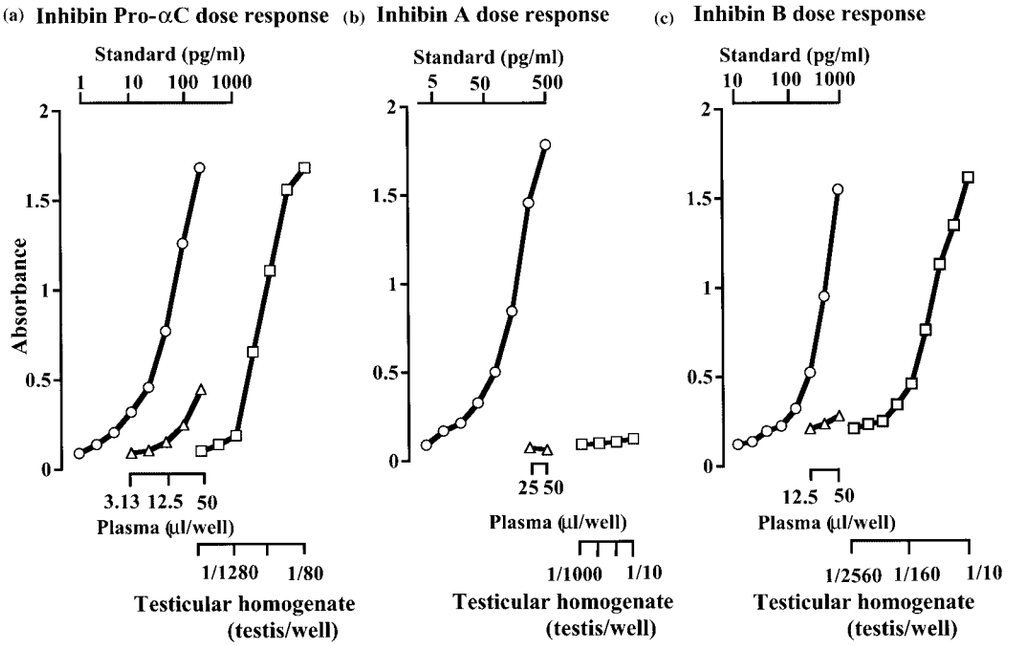

Figure 2. Dose-dependent curves of inhibin pro-αC **(a)**, inhibin A **(b)**, and inhibin B **(c)** in peripheral plasma (△) and testicular homogenate (□) of male golden hamster produced with human inhibin standard (○).

Sertoli cells were strongly stained for inhibin α subunit, suggesting that Sertoli cells may secrete inhibin α subunit or inhibin pro-αC.

Results of the ELISA in the present study demonstrated that the dose-dependent curves produced by serially diluted peripheral plasma and testicular homogenates were paralleled to the standard curves produced with both inhibin B and inhibin pro-αC. However, parallelism was not observed with the standard curve produced with inhibin A. Plasma concentrations of inhibin pro-αC were reduced below the limit of assay detection, and inhibin B also fell significantly after bilateral orchidectomy, indicating that inhibin pro-αC and inhibin B are mainly secreted by the testes in the male golden hamster. The present findings, therefore, suggest that inhibin B is the major form of dimeric inhibin secreted by the testes of adult male golden hamsters. Furthermore, the very low circulating level of inhibin A reported in the present study agrees with findings of others (Illingworth et al, 1996; Woodruff et al, 1996). Previous studies have suggested that inhibin B may have a role in the development of spermatogonia in the testes of adult male animals (Foppiani et al, 1990; Anderson et al, 1998; Ramaswamy et al, 2000).

In conclusion, present results demonstrate that the inhibin α and βB subunits are localized in the Leydig cells of the testes in the adult male golden hamster. These inhibin subunits are colocalized with 3β-HSD in the same Leydig cell population. It is therefore suggested that inhibin B is mainly secreted by Leydig cells of the testes in the adult male hamster.

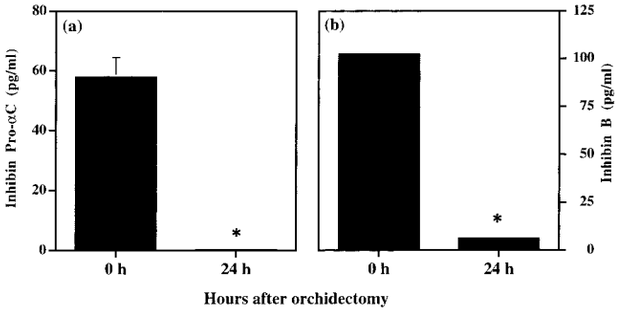

Figure 3. Effects of orchidectomy on plasma concentrations of inhibin Pro-αC **(a)** and inhibin B **(b)**. Each value represents mean ± SEM of 5 animals. *$P < .001$ compared with 0-hour value (Student's *t* test). Note that in panel **(b)**, SEM for inhibin B at 0 and 24 hours were ± 0.011 pg and ± 0.004 pg, respectively.

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