Ontogeny of inhibin secretion in the rat testis: secretion of inhibin-related proteins from fetal Leydig cells and of bioactive inhibin from Sertoli cells

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Abstract
The ontogeny of inhibin secretion in the testis of rats was investigated. Testicular localization, content of immunoactive and bioactive inhibin and its molecular size in fetal and neonatal rats (from 16 days of gestation to 5 days of age) were determined. Strong immunostaining with an antiserum against a polypeptide of porcine inhibin α-subunit was noted in testicular interstitial cells from 16 days of gestation. Co-localization of inhibin α-subunit and 3β-hydroxysteroid dehydrogenase (3βHSD) was observed in the interstitial cells until 2 days of age. Immunoreactive inhibin α-subunit in the interstitial tissue had disappeared by 5 days of age, although 3βHSD-positive cells were still detected. Weak immunostaining for the inhibin α-subunit was detected in the seminiferous tubules, probably in the cytoplasm of Sertoli cells, from 20 days of gestation onward. No inhibin α-subunit immunostaining was observed in germ cells throughout the experimental period. Testicular inhibin was detected at 16 days of gestation (49.5 ± 6.7 pg per testis) by RIA. Testicular immunoreactive inhibin showed a tendency to increase during fetal life and levels were maintained at a similar value after birth (697.0 ± 46.9 pg per testis at 5 days of age). Inhibin bioactivity and its molecular size in testicular homogenate was examined at 17 days of gestation and 0 and 5 days of age. Although no bioactivity was detected at 17 days of gestation, bioactivity was noted at 0 and 5 days of age (177.7 and 1303.9 pg per testis respectively). Immunoblot analysis with antiserum against inhibin α-subunit revealed only approximately 40 kDa molecular masses in the tests at 17 days of gestation, probably inhibin-related proteins, but not inhibin. At 0 and 5 days of age, a protein of 30 kDa molecular mass, possibly inhibin, was detected as well as material of approximately 40 kDa molecular mass. FSH in the plasma was first detected at 19 days of gestation (1197.0 ng/l), increased towards birth, and thereafter decreased (4588.5 ± 572.3 ng/l at 21 days of gestation and 2400.0 ± 179.6 ng/l at 5 days of age). These results indicate that Leydig cells in fetal and neonatal rats produce inhibin-related substances with no inhibin bioactivity, whereas Sertoli cells begin to produce inhibin during the perinatal period as a possible regulator of FSH secretion.

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Introduction
Inhibin is a dimeric gonadal glycoprotein which consists of an α-subunit and a β-A- or β-B-subunit, and selectively suppresses follicle-stimulating hormone (FSH) secretion from the anterior pituitary gland. Homodimers of β-subunits called activin stimulate FSH secretion. Inhibin-related proteins, several large forms of inhibin and precursors of the α-subunit have been identified in bovine follicular fluids (Robertson et al. 1989) and in fetal bovine testicular extracts (Torney et al. 1992). Their cross-reactivity with antiserum against inhibin in RIA (Robertson et al. 1989) raised the problem that levels of inhibin measured by RIA could not fully explain the suppressive effects of inhibin on FSH secretion. In addition to its role in FSH secretion, the role of inhibin as a local regulator of spermatogenesis has been indicated in rats (Franchimont et al. 1981, Bhasin et al. 1989, Gonzales et al. 1989, Hakovirta et al. 1993) and hamsters (van Dissel-Emiliani et al. 1989).

The major source of inhibin secretion is Sertoli cells in male animals. Although testicular secretion of inhibin and its role in the regulation of FSH secretion have been shown in rats during the postnatal period (Hermans et al. 1980, Au et al. 1986, Ultee-van Gessel & de Jong 1987, Culler & Negro-Vilar 1988, Rivier et al. 1988), the ontogeny of
immunoblot of inhibin of three pregnant females and five neonatal male rats after chemicalexamination, testes were collected from embryos designated as being 0 days of age. For immunohisto-

determination to examinewhetherareciprocal

periods using immunohistochemistry, bioassays, and immunoblot techniques. Concentrations of FSH in the plasma were determined to examine whether a reciprocal

relationship between secretion of FSH and inhibin could be detected during these periods.

Materials and Methods

Samples

Fetal and neonatal Wistar rats from 16 to 21 days of gestation and from 0 to 5 days of age were used. Fetuses were designated as being 0 days of gestation when sperm were detected in the vaginal smear of the mother. Rats born in the afternoon of the 21st day of gestation were designated as being 0 days of age. For immunohistochemical examination, testes were collected from embryos of three pregnant females and five neonatal male rats after decapitation. For bioassay and RIA of inhibin and for immunoblot of inhibin α-subunit, blood and testes were collected from embryos of five to ten pregnant females and from neonatal rats of five dams after decapitation. From 16 to 19 days of gestation, all the blood of embryos at the same day of gestation was pooled in order to obtain a large enough volume for assay. From 20 days of gestation onward, blood of embryos or infants from each mother was pooled. Testes of embryos or infants from each mother were collected and pooled from 16 days of gestation onward. They were homogenized in 0·5 ml 0·85% (w/v) NaCl and centrifuged at 36000 g for 30 min. Supernatants were collected and stored at −20 °C until assays were performed.

Immunohistochemistry of inhibin α-subunit and 3β-hydroxysteroid dehydrogenase (3βHSD)

Testes were fixed with Methacarn solution (methanol–chloroform–acetic acid, 6:3:1, by vol) and embedded in paraffin. Serial 3 µm sections were cut. After being deparaffinized, sections were immersed in 6 m urea for 30 min at room temperature, and then incubated in 0·5% (w/v) periodic acid to block endogenous peroxidase. Sections were incubated overnight at 4 °C with antiserum against inhibin α-subunit or antiserum against 3βHSD. ABC kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine was used for visualizing. In the immunostaining experiments, all sections from each age group were examined at the same time. All the experiments were repeated at least three times. Antiserum against synthetic inhibin α-subunit(1–30) conjugated with rabbit albumin (provided by Dr N Ling, Neurocrine Biosciences Inc., La Jolla, CA, USA) was raised in rabbits. According to the specificity of the antiserum, there was evidence of cross-reactivity with 58 and 32 kDa bovine inhibin and inhibin–related peptide (25 kDa) and potency to immunoneutralize inhibin activity in the rat both in vivo and in vitro (using the rat pituitary cell bioassay system described below; data not shown). The antiserum was used at a dilution of 1:1000. It was considered to be a specific reaction when immunopositive reactions were diminished after the addition of absorbed antiserum treated as follows. Purified bovine inhibin was mixed with the antiserum to a final concentration of 20 g/l and incubated overnight at 4 °C. After centrifugation at 1700 g for 30 min at 4 °C, supernatant was used as the absorbed antiserum at the same dilution as for immunohistochemical staining. Antiserum against 3βHSD was supplied by Dr J J Mason (Cecil H and Ida Green Center for Reproductive Science, University of Texas, Southern Medical Center, Dallas, TX, USA). Specifications for the antiserum have been described previously (Doody et al. 1990, Sasano et al. 1990).

RIAs

Concentrations of FSH in the serum were measured by double-antibody RIA using NIDDK RIA kits for rat FSH (NIAMDD, NIH, Bethesda, MD, USA). The iodinated preparation was rat FSH-I-5 and the antiserum was anti-rat FSH-S-11. Results were expressed in terms of NIDDK rat FSH-RP-2. The intra- and interassay coefficients of variation were 9·2 and 9·9% respectively. Immunoreactive inhibin in the serum and testes was also measured by double-antibody RIA using a rabbit anti-

serum (TNDH-1), which was generated using partially purified bovine inhibin prepared by immunoaffinity chromatography and iodinated 32 kDa bovine inhibin as described previously (Hamada et al. 1989). The intra- and interassay coefficients of variation were 8·5 and 13·2% respectively. Results are expressed as pg of 32 kDa bovine inhibin.

Inhibin bioassay

Bioactivity of inhibin in testicular homogenates at 17 days of gestation and 0 and 5 days of age was determined using
the rat pituitary cell bioassay system as described previously (Taya & Sasamoto 1988). The standard employed was the same preparation as used in the inhibin RIA. The potency of this bovine reference preparation in relation to the WHO porcine inhibin reference preparation (86/690) is equivalent to 0·156 U 86/690 per pg of our reference preparation.

**Immunoblotting of inhibin α-subunit**

Testicular homogenates at 17 days of gestation and 0 and 5 days of age were subjected to SDS–PAGE as described by Laemmli (1970). The amounts of immunoreactive inhibin in the applied testicular homogenate were 138·0 and 107·5 pg per lane at 17 days of gestation, 157·1 pg per lane at 0 days of age and 152·0 pg per lane at 5 days of age. Partially purified bovine inhibin preparation after immunoaffinity chromatography was used as standard. Samples were mixed with equivalent volumes of buffer containing 4% SDS with (reduced) or without (non-reduced) 10% β-mercaptoethanol. For reduction, the mixed samples were heated in a boiling water bath for 2 min. SDS–PAGE was carried out in 12% and 10% acrylamide slab gels under reducing conditions and in 15% and SDS–PAGE was carried out in 12% and 10% acrylamide slab gels under non-reducing conditions. Proteins on the gel were transferred to an Immobilon membrane (polyvinylidine difluoride membrane; Millipore, Bedford, MA, USA) at 2 mA per cm² for 2 h using Millipore transblot apparatus. The membrane was incubated overnight with antiserum against inhibin α-subunit at a dilution of 1:500 at room temperature, then with peroxidase-labelled anti-rabbit immunoglobulin (Bio-Rad Laboratories, Hercules, CA, USA). POD immunostaining kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used for visualizing the immunoreaction. All the experiments were repeated three times or more. It was considered to be a specific reaction when immunopositive reactions were diminished by absorbed antiserum. For absorption controls, the absorbed antiserum prepared as for the immunohistochemistry was used at a dilution of 1:500.

**Results**

**Testicular localization of inhibin α-subunit and 3βHSD (Table 1 and Fig. 1)**

A summary of immunostaining in the testis with antiserum against a polypeptide of porcine inhibin α-subunit is shown in Table 1. Seminiferous tubules were surrounded by a single layer of flat elongated cells and were distinct from the interstitial tissue at 16 days of gestation. The tubules contained two cell types: germ cells, larger ones with a spherical nucleus, and Sertoli cells with nuclei of various size. The interstitial tissue was abundant and several types of cell were present in this tissue. Cells with a small round nucleus or a larger oval nucleus were observed. Strong staining with the antiserum against inhibin α-subunit was detected in the former type of interstitial cell at 16 days of gestation (Fig. 1B). These inhibin α-subunit-positive interstitial cells were also positively stained with antiserum against 3βHSD in the serial section (Fig. 1C). No immunostaining with either antiserum was observed in the tubules.

In the testis of perinatal rats, germ cells were located in the central region of the tubule and Sertoli cells were located along the basement membrane. Mitotic figures of Sertoli cells were observed within the tubules from 19 days of gestation onward, indicating proliferation of Sertoli cells. The strong immunostaining with both the antiserum against inhibin α-subunit and that against 3βHSD was still observed in the same interstitial cells. Weak immunostaining for inhibin α-subunit was also noted in the tubules from 19 days of gestation onward (Fig. 1D), which appeared to be in the cytoplasm of Sertoli cells. No immunostaining was observed in the cytoplasm of gonocytes.

In the testis of neonatal rats, seminiferous tubules contained numerous Sertoli cells, the nuclei of which were adjacent to the basement membrane. Gonocytes were located in the central region of the tubule though some gonocytes were observed to be located near the basement membrane, among the Sertoli cells. The interstitial tissue was less abundant than in fetal testes. Immunostaining for inhibin α-subunit in the interstitial cells had mostly disappeared by 5 days of age (Fig. 1F), although intensely immunostained cells for 3βHSD were still present in the interstitial tissue (Fig. 1G).

<table>
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<th>Table 1 Immunohistochemical localization of inhibin α-subunit in the testis of fetal and neonatal rats</th>
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<td>++++, Strong staining; ++, moderate staining; +, weak staining; −, undetectable.</td>
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Testicular content of immunoreactive inhibin (Fig. 2) and bioactive inhibin

Immunoreactive inhibin was detected in the testis of rats from 16 days of gestation (49.5 ± 6.7 pg per testis).

Testicular content of immunoreactive inhibin increased up to 19 days of gestation, and these levels were maintained until 21 days of gestation. The content of immunoreactive inhibin in the testis further increased up to 0 days of age.
and the levels remained steady until 5 days of age. Bioactivity of testicular inhibin was examined at 17 days of gestation and 0 and 5 days of age. Bioactivity was not detected at 17 days of gestation, despite the presence of immunoreactive inhibin (132.2 pg per testis). The average contents of bioactive and immunoreactive inhibin in the testis were 177.7 and 626.6 pg per testis at 0 days of age and 1303.9 and 621.4 pg at 5 days of age respectively. Therefore the corresponding ratio of biological and immunological activities of inhibin at 5 days of age showed a 7.6-fold increase over that at 0 days of age.

**Immunoblot analysis of testicular inhibin** (Fig. 3)

Immunoblot showed the presence of different molecular mass forms of inhibit α-subunit-related proteins. Under non-reducing conditions, 43 and 44 kDa proteins were detected in the testis at 17 days of gestation, but 30 kDa protein was not detected. In addition to the several approximately 40 kDa bands, a 30 kDa protein was also detected faintly at 0 days of age and this band became clear at 5 days of age. When the sample at 17 days of gestation was reduced, slightly higher molecular mass forms (47 to 50 kDa) were detected than those observed under non-reducing conditions. At 0 and 5 days of age, the 30 kDa protein detected in the non-reduced sample disappeared, whereas 16 and 20 kDa proteins appeared in addition to the 47 to 50 kDa proteins, when reduced. On immunoblotting the non-reduced sample at 5 days of age, a 45 kDa band was observed when reacted with the absorbed...
antiserum. Therefore bands of this molecular mass were not considered to be inhibin-related protein.

Concentrations of FSH and immunoreactive inhibin in the serum (Fig. 4)

FSH was first detected in the serum at 19 days of gestation (1197·0 ng/l). Concentrations of FSH in the serum increased markedly until birth (4588·5 ± 572·3 ng/l at 21 days of gestation) but decreased during the neonatal period. Immunoreactive inhibin was detected in the serum of rats from 16 days of gestation (153·8 ng/l) onward and the concentration was between 200 and 400 ng/l throughout the fetal and neonatal periods.

Discussion

Our immunohistochemical study clearly shows strong immunostaining for inhibin α-subunit in the testicular interstitial cells in which immunostaining for 3βHSD was also detectable during the fetal and neonatal period. Neither inhibin bioactivity nor bioactive molecular forms of inhibin were noted in the testicular homogenate of fetal rats. These results indicate that Leydig cells secrete inhibin-related proteins but not inhibin during this period.

Although several reports have demonstrated the possibility of secretion of inhibin-related proteins from normal (Roberts et al. 1989, Risbridger et al. 1989, Bergh & Cajander 1990, Rabinovici et al. 1991, Teeds et al. 1991, de Winter et al. 1992) and tumour (Bergh & Cajander 1990, de Jong et al. 1990, de Winter et al. 1992, Noguchi et al. 1993) Leydig cells, the suppressive effect of these proteins on FSH secretion has not been consistently demonstrated. Lee et al. (1989) detected stimulation of FSH secretion in the culture medium of Leydig cells of immature rats. In fetal rats, immunohistochemical studies (Koike & Noumura 1993) as well as expression of inhibin α-subunit mRNA (Roberts et al. 1991) suggested the secretion of inhibin-related proteins from testicular interstitial cells. However, Shaha et al. (1989) reported the production of inhibin α-subunit in the seminiferous tubules but not in the interstitial cells.

In the present study, regardless of immunoreactive inhibin, no inhibin bioactivity was detected in the fetal testicular homogenate. Since we used a pituitary cell bioassay system, the result could have been affected by the co-presence of inhibin, activin or follistatin. Immunoblot analysis in the fetal testis revealed the presence of inhibin-related proteins of molecular mass approximately 40 kDa, but not bioactive forms of inhibin as previously reported by de Kretser & McFarlane (1996). A 30 kDa molecular mass was detected in the testis after birth and this molecule had a 20 kDa form stained with α-subunit-specific antiserum under reducing conditions, which was considered to be inhibin α-subunit. The 16 kDa immunoreactivity detected together with the 20 kDa band appears to be due to the loss of several amino acids at the N-terminus of the 20 kDa inhibin α-subunit. From these results, the 30 kDa molecule is thought to be a mature form of inhibin, and the absence of both bioactivity and bioactive molecular forms of inhibin in the testis of fetal rats supports the idea that fetal Leydig cells produce inhibin-related proteins which lack inhibin bioactivity.

Immunostaining for inhibin α-subunit in Leydig cells faded during the neonatal period, which is consistent with the results of Koike & Noumura (1993). The reason for the change in the degree of immunoreactivity observed
in Leydig cells is unclear. Two marked phases of Leydig cell growth and a shift from the fetal to the adult type within the first 3 weeks after birth have been reported (Roosen-Runge & Anderson 1959, Lording & de Kretser 1972). On the other hand, germ cells (gonocytes) are known to start differentiation into spermatogonia, migrating from the central to the basal area in the seminiferous tubule, in the first week after birth (Gondos 1977). The change in the degree of inhibin-related protein production by Leydig cells could be related to the onset of spermatogenesis. Direct effects of inhibin on germ cell proliferation during the cycle of the seminiferous epithelium has been shown in rats and other animals (Franchimont et al. 1981, van Dissel-Emiliani et al. 1989, Hakovirta et al. 1993), whereas the role of inhibin-related proteins in the regulation of spermatogenesis, especially at the beginning of spermatogenesis, has not been determined. Further investigations are necessary to clarify the role of these proteins in spermatogenesis.

Inhibin bioactivity as well as a bioactive form of inhibin (30 kDa) in the testicular homogenate was detected only after birth. Therefore inhibin could be a regulator of FSH secretion during the neonatal period, but not during the fetal period in the male rat. A marked increase in inhibin bioactivity in the testicular homogenate between 0 and 5 days after birth is in agreement with previous reports (Au et al. 1986, Ultee-van Gessel & de Jong 1987). The immunostaining for inhibin α-subunit was detected in Sertoli cells from the perinatal period, whereas that in Leydig cells had disappeared by 5 days after birth. These results indicate that an increase in testicular inhibin bioactivity can be attributed to mature inhibin derived from Sertoli cells. The increase in testicular inhibin bioactivity possibly results in an increase in circulating levels of bioactive inhibin, because the testis is a major source of inhibin secretion in male animals. A decrease in serum levels of FSH during the neonatal period is probably, at least in part, due to suppression of FSH secretion by inhibin derived from Sertoli cells. Culler & Negro-Vilar (1988) observed an increase in circulating levels of FSH after immunoneutralization of inhibin in neonatal male rats. Sertoli cells showed a decrease in active proliferation during this period, and proliferation could be stimulated by FSH (Griswold et al. 1977, Orth 1982, 1984). The reciprocal relationship between secretion of bioactive inhibin and proliferation in Sertoli cells suggests that Sertoli cells regulate their own number by secretion of inhibin through modulation of FSH secretion. Further investigations aimed at clarifying this point are continuing in our laboratory.

In the present studies, FSH in the serum was first detected at 19 days of gestation, which is consistent with previous reports (Tougard et al. 1977, Watanabe & Daikoku, 1979, Huhtaniemi 1995). However, this is inconsistent with the report by Chowdhury & Steinberger (1976), who detected high levels of FSH in the plasma from 16 days of gestation. The reason for this discrepancy is not clear.

In conclusion, we have demonstrated that Leydig cells secrete inhibin α-subunit-related proteins during fetal life, while Sertoli cells start to secrete mature inhibin during the perinatal period, as a regulator of FSH secretion. Further investigations are necessary to clarify the role of inhibin-related proteins from Leydig cells during the fetal and perinatal periods.

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