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Inhibins in the Male Göttingen Miniature Pig: Leydig Cells Are the Predominant Source of Inhibin B

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ABSTRACT: The expression of inhibin subunits in the testes of the Göttingen miniature pig was examined by in situ hybridization and immunohistochemistry. In addition, the major forms were determined by enzyme-linked immunosorbent assay (ELISA). Strong positive immunostaining for the inhibin α subunit was observed in Sertoli and late-stage germ cells, but it was weak in Leydig cells. However, Leydig cells showed strong positive staining for the βA subunit, but Sertoli cells and spermatogonia showed a weak reaction. Strong positive immunostaining for the βB subunit was observed in Leydig cells but spermatogonia showed weak staining for it. In contrast to the staining specificity of inhibin α and βA subunits, the βB subunit did not exhibit positive staining in Sertoli cells. In situ hybridization revealed that although the α subunit mRNA signal was highly expressed in all cell types, the reaction appeared to be stronger in Sertoli cells and spermatogonia than in Leydig cells. βA subunit mRNA expression was somewhat identical to that of the α subunit, however, germ cells showed a weak stain for it. A strong, positive mRNA signal for the βB subunit was confined to Leydig cells and late-stage germ cells. ELISA results showed that concentrations of inhibin B and inhibin pro-αC were high in the circulation and testes. In contrast, inhibin A levels in both plasma and testes were undetectable. The present results strongly suggest that inhibin B is the major form of circulating inhibin and that Leydig cells are the predominant source of this dimeric inhibin in male Göttingen miniature pigs. Furthermore, the germ cells also appear to be an important source of circulating inhibins.

Key words: ELISA, immunohistochemistry, in situ hybridization, Sertoli cell, testes.

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Inhibin is a heterodimeric protein consisting of an α subunit and one of two β subunits. Two related forms of inhibin, inhibin A (α/βA) and inhibin B (α/βB) are secreted into the circulation by the gonads and inhibit pituitary secretion of follicle-stimulating hormone (FSH; Ling et al, 1985; Miyamoto et al, 1985; Rivier et al, 1985; Robertson et al, 1985; Vale et al, 1986). It is generally accepted that in adult males, Sertoli cells are the major source of testicular inhibins (Maddocks et al, 1989; Roberts et al, 1989; Shaha et al, 1989). Inhibin has also been shown to exert paracrine and/or autocrine effects within the gonads (Lin et al, 1989; Chen, 1993), and functions as a paracrine regulator in spermatogenesis in mice (van Dissel-Emailiani et al, 1989), hamsters (van Dissel-Emailiani et al, 1989), and rats (Mather et al, 1990; Kaipia et al, 1992). A recently developed specific immunoassay has enabled measurement of plasma levels of dimeric inhibins and provided evidence that inhibin B is an important physiological form of inhibin in males (Illingworth et al, 1996; Foppiani et al, 1999; Sharpe et al, 1999; Ramaswamy et al, 2000; Jin et al, 2001). It has also been suggested that in human men, inhibin B regulates FSH secretion (Anawalt et al, 1996; Illingworth et al, 1996; Nachtigal et al, 1996; Seminara et al, 1996), and plasma concentration of inhibin B is closely related to the number of sperm in the ejaculate (Jensen et al, 1997) and testicular volume (Pierik, 1998). Based on these observations, it has been suggested that inhibin B may be a good marker for spermatogenesis.

The Göttingen miniature (GM) pig is often considered to be a potential donor for organ transplantation in humans (Maki et al, 1996). Thus, this European breed is an important species for study. However, it has a considerably smaller litter size than the Chinese pig (Haley et al, 1993). A thorough understanding of the GM pig's reproductive hormonal profile is necessary in order to increase its breeding performance. However, the reproductive hormonal profiles including that of heterodimeric inhibins and their cellular source have not been documented in GM pigs.

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Thus in the present study, testicular localization of inhibin α, βA, and βB subunits were investigated using immunohistochemistry. Furthermore, in situ hybridization was used to identify messenger RNAs for all three inhibin subunits in the testes of GM pigs. In order to determine the major form of inhibins, plasma concentrations of inhibin pro-αC, inhibin A, and inhibin B were measured using two-site enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Animals and Samples

Ten adult male GM pigs (3 to 9 months old) were used in this study. Blood samples were collected by jugular venipuncture into heparinized centrifuge tubes, and immediately centrifuged at 1700 × *g* for 15 minutes at 4°C. Plasma was separated and stored at −20°C until assayed for hormones. Ten testes were collected, homogenized using a homogenizer (Histocoron; Nichion Ltd, Tokyo, Japan) in 1 mL of 0.85% NaCl (w/v), followed by centrifugation at 20 800 × *g* for 30 minutes. The supernatant was separated and stored at −20°C until used in the assay. Another 10 testicular samples were prepared for immunohistochemistry and in situ hybridization. The tissue samples were immediately fixed in 4% paraformaldehyde (Sigma Chemical Co, St Louis, Mo) in 0.05 M phosphate-buffered saline (PBS) pH 7.4, and embedded in paraffin. The paraffin-embedded testicular tissues were serially sectioned at a thickness of 6 μm and placed on poly-L-lysine (Sigma Diagnostics, Inc) coated slide glasses (Dako Japan Co, Kyoto, Japan) and 3-amino propyltriethoxy silane (APS) coated slide glasses (Matsunami Glass Ind, Ltd, Japan) for use in immunohistochemistry and in situ hybridization, respectively.

All experimental procedures involving animals were carried out in accordance with the Tokyo University of Agriculture and Technology *Guide for the Care and Use of Laboratory Animals.*

Enzyme-Linked Immunosorbent Assay

Concentrations of inhibin A, inhibin B, and inhibin pro-αC in plasma and testicular homogenates were measured using a two-site ELISA kit specific for each peptide (Serotec Ltd, Oxford, United Kingdom). The characteristics of these inhibin dimer-specific assays have been previously described (Groome et al, 1994).

Immunohistochemistry for Inhibin α, βA, and βB Subunits

After deparaffinized with xylene, the tissue sections 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 7.5% H_2O_2 in methanol at room temperature for 1 hour 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. Then they were incubated at 37°C for 16–18 hours with polyclonal antibodies made against inhibin subunits at dilutions of 1:8000 to 1:16 000 in CTS. The antibody against each inhibin subunit was anti-[Tyr30]-porcine inhibin α chain (1-30)-NH_2 conjugated to rabbit serum albumin, acetyl anti-porcine inhibin βA (81-113)-NH_2 (#305-24D), and anti-cyclic acetyl human inhibin βB (80-112)-NH_2 (#305-25D). The use of these antibodies has been described by Meunier et al (1988a). After incubation with specific antibodies, 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. These sections 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 them with 0.025% (w/v) 3,3′-diaminobenzidine tetrachloride (DAB; Sigma Chemica Co, St Louis, Mo) in 100 mM Tris-buffered saline containing 0.01% H_2O_2 for 1–30 minutes. Sections were counterstained with hematoxylin for 1 second to better visualize the cell types.

Probe Preparation

The complementary DNAs (cDNAs) for the 3 porcine inhibin subunits (α, βA, and βB) were kindly provided by Dr M.D. Li (Molecular Resource Center, University of Tennessee). Preparation of these cDNAs has been previously described (Li et al, 1997). The cDNAs were transcribed to synthesize RNAs using an SP6/T7 transcription kit (Boehringer-Mannheim, Mannheim, Germany). The sense and antisense probes were prepared using digoxigenin (DIG) RNA Labeling Mixture (Boehringer-Mannheim). The antisense and sense were diluted to a concentration of 20 ng/100 mL in hybridization buffer made of 50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, transfer RNA 0.5 mg/mL, 1× Denhardts solution, and 2.5 mM ethylenediamine tetraacetic acid (pH 8.0).

In Situ Hybridization for Porcine Inhibin Subunits

Sections were deparaffinized with xylene and washed in 10 mM PBS buffer. They were incubated in 0.2 N HCl for 15 minutes and washed in 100 mM PBS. Sections were digested with protease (10 μg/mL; Sigma) in 100 mM PBS for 20 minutes at 37°C and washed in 100 mM PBS for 5 minutes, rinsed again in 10 mM PBS for 10 minutes followed by sequential dehydration through a graded series of alcohol, and air-dried. Hybridization was performed by covering the sections with approximately 200 μL of diluted probe and incubating them overnight at 45°C. Following overnight incubation the sections were washed thoroughly in formamide buffer (50% formamide, 40% distilled water, and 2× saline-sodium citrate [SSC]) for 1 hour at 50°C. They were rinsed copiously in RNase buffer (0.5 M NaCl and 10 mM Tris-HCl pH 8.0) and digested with RNase A (20 mg/mL; Sigma) for 15 minutes at 37°C to remove the unhybridized riboprobe. After digestion the sections were washed in RNase buffer again, and rinsed in 1× SSC/50% formamide buffer for 30 minutes at 50°C. This was followed by washing the sections in buffer 1 (100 mM Tris-HCl/150 mM NaCl pH 7.5) twice for 10 minutes, and blocking them in buffer 2 (0.5% blocking reagent in buffer 1) for 1 hour at room temperature. The sections were then incubated with polyclonal anti-DIG (Fab fragment conjugated to alkaline phosphatase) (1:500 dilution) for 1 hour at room temperature, and washed in buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) three times

for 15 minutes. Messenger RNA signals of three inhibin subunits (α, βA, and βB) were detected by a colorimetric method using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Promega, Madison, Wis).

Statistics

Dilutions of plasma and testicular homogenates were used in the inhibin ELISA assays to check for parallelism with human inhibin standard by 2 × 2 point analysis (Finney, 1964).

Results

Immunohistochemistry

In the testicular sections of the adult male GM pig, a strong positive immunostaining for the inhibin α subunit was observed within the peritubular cells, presumably in the cytosol of Sertoli cells, whereas positive staining in Leydig cells appeared weak (Figure 1B). Positive immunostaining for the inhibin α subunit in late-stage germ cells was also observed (Figure 1B). Leydig cells were positively stained for both the inhibin βA (Figure 1C) and βB (Figure 1D) subunits, whereas the immunostaining reaction for the βA subunit was weak in Sertoli cells. Furthermore, a weak immunostaining reaction for both the inhibin β subunits was detected in spermatogonia. Sections incubated with normal rabbit serum instead of primary antibody did not show any immunopositive staining (Figure 1A).

In Situ Hybridization

Messenger RNAs for porcine inhibin α, βA, and βB subunits were localized by nonradioactive in situ hybridization using DIG-labeled inhibin antisense probes as the positive hybridization probe and the corresponding sense probes as the negative probe. Inhibin α subunit mRNA signal was highly expressed in Sertoli (Figure 2A, arrowheads) and peritubular cells and spermatogonia, whereas the signal in Leydig cells was relatively weak (Figure 2A). Inhibin βA subunit mRNA expression was somewhat identical to that of the α subunit; however, the Sertoli cell appeared to be the major cell type that expressed the βA subunit mRNA signal (Figure 2C). Messenger RNA signal for the βB subunit was localized primarily to Leydig cells and also to some late-stage germ cells (Figure 2E). There were no positive signals for the α (Figure 2B), βA (Figure 2D), or βB (Figure 2F) subunits in sections treated with sense probes.

Plasma Concentrations of Inhibin A, Inhibin B, and Inhibin Pro-αC

Dose-dependent curves of serially diluted samples of peripheral plasma and testicular homogenates were parallel to the standard curves produced with inhibin pro-αC (Figure 3a) and inhibin B (Figure 3c), whereas plasma and testicular homogenates failed to show parallelism with the standard curve produced with inhibin A (Figure 3b), suggesting that inhibin A was not measurable in either peripheral plasma or testicular homogenates. Concentrations of inhibin pro-αC and inhibin B in peripheral plasma and testicular homogenates were measured and are summarized in the Table.

Discussion

This is the first report to show that the major form of circulating inhibins in male GM pigs is inhibin B, and that it is produced mainly by Leydig cells and to some extent by germ cells. The distribution of three inhibin subunits in the testes of male GM pigs was analyzed using immunohistochemistry and in situ hybridization. It is interesting that the βB subunit was clearly immunolocalized in Leydig cells and some late-stage germ cells, but not in Sertoli cells, although α and βA subunits were detected in all cell types. Furthermore, in situ hybridization analysis of mRNAs showed a similar expression pattern of α, βA, and βB subunits in Leydig cells and Sertoli cells. These observations indicate a species-specific cellular distribution of inhibins in male GM pigs. We have previously shown that Leydig cells are the main producers of inhibin B in male hamsters (Jin et al, 2001), and others have reported that a majority of inhibin βB subunit was localized to fetal-type Leydig cells in testes of fetal and neonatal humans and rats (Majdic et al, 1997). It has also been reported that both Leydig and Sertoli cells contribute to circulating inhibin B levels in stallions (Nagata et al, 1998) and human adults (Anderson et al, 1998). Recently, Sharpe et al (1999) reported that inhibin B is largely produced by Sertoli cells of the testes in the rat. Thus, it seemed reasonable to suggest that the cellular source of testicular inhibin B secretion is species-specific. The present immunohistochemical data show that the α subunit is stained in Leydig and Sertoli cells, but the βB subunit is localized to Leydig cells only. This agrees with present in situ hybridization data showing that the intensity of α subunit signals is higher in Sertoli cells than in Leydig cells. However, the data from both techniques indicate that strong expression of inhibin βB protein and its mRNA are confined to the Leydig cells, but not to the Sertoli cells. Furthermore, the ELISA findings of detectable inhibin B levels as opposed to undetectable inhibin A levels in plasma and testicular homogenates suggest that inhibin B is the major form of dimeric inhibin in male GM pigs. This agrees with findings of several previous studies that inhibin B is the major form of dimeric inhibin in men (Anawalt et al, 1996; Illingworth et al, 1996), male monkeys (Plant et al, 1997; Foppiani et al,

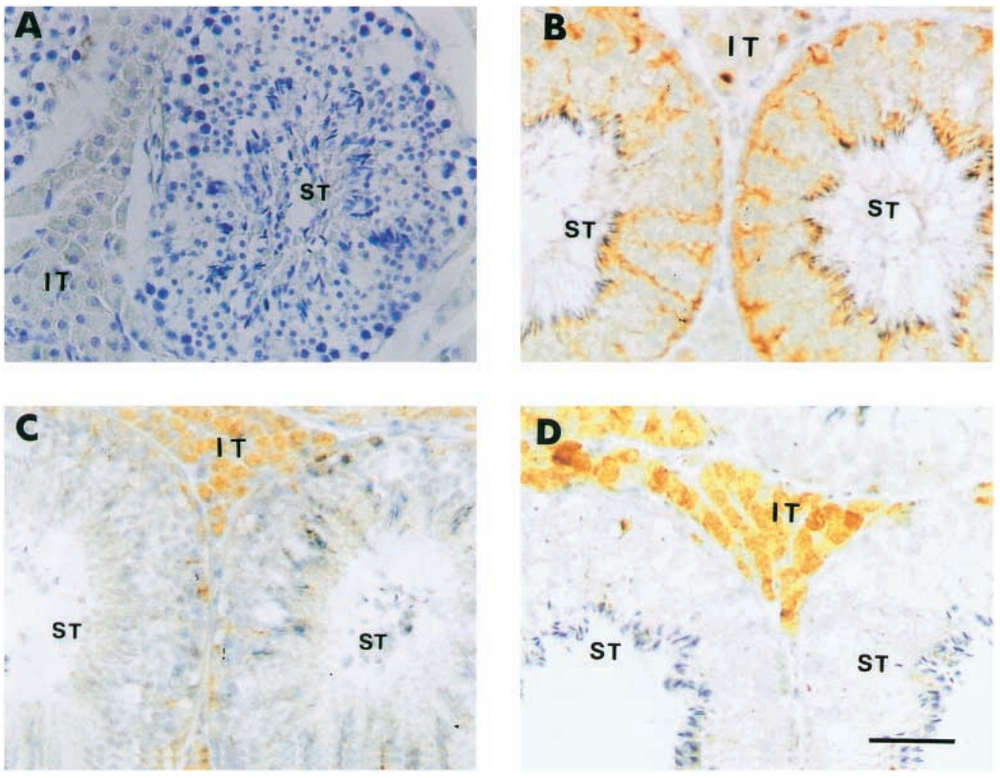

Figure 1. Immunohistochemistry for the three inhibin subunits in Göttingen miniature pig testes. (**A**) Stained with normal rabbit serum (NRS). **(B–D)** Stained with anti-inhibin α, βA, and βB subunits, respectively. All sections were counterstained with hematoxylin. ST indicates seminiferous tubule; IT, interstitial tissue. Bar = 50 μm.

1999; Ramaswamy et al, 2000), male rats (Woodruff et al, 1996), and male golden hamsters (Jin et al, 2001).

Although inhibin A levels in both the circulation and testicular homogenates were below the limit of detection, the immunohistochemical and in situ hybridization findings indicate that Sertoli, Leydig, and germ cells can produce inhibin A in this species. These findings also agree with previous results by others who reported that mRNA levels for the inhibin βA subunit were either very low or below the limit of detection in the rat testis (Esch et al, 1987; Meunier et al, 1988b; Bhasin et al, 1989; Feng et al, 1989). In a previous study of testes of the male golden hamster (Jin et al, 2001), we did not detect inhibin A in peripheral plasma nor did we observe βA subunit immunostaining. On the other hand, the βA subunit protein has been detected in Sertoli cells and isolated interstitial cells, and an mRNA signal has been detected in isolated interstitial cells of the rat testis (Roberts et al, 1989). In addition, Kaipia et al (1992) reported that expression of inhibin βA subunit mRNA was highly stage-specific in Sertoli cells. It was therefore reasonable to suggest that inhibin A may have some local function in the testis because its levels in the circulation are very low.

On the other hand, in GM pigs, the germ cells of the testes appeared to be an important source of circulating inhibins. The present results show that germ cells exhibit staining for all three inhibin subunits and suggest that the cells may secret inhibin pro-αC or dimeric inhibins. Furthermore, the data strongly support findings by others who demonstrated that the three inhibin subunits were positively stained in spermatogenic cells at multiple stages of development (Bhasin et al 1989; Shaha et al, 1989;

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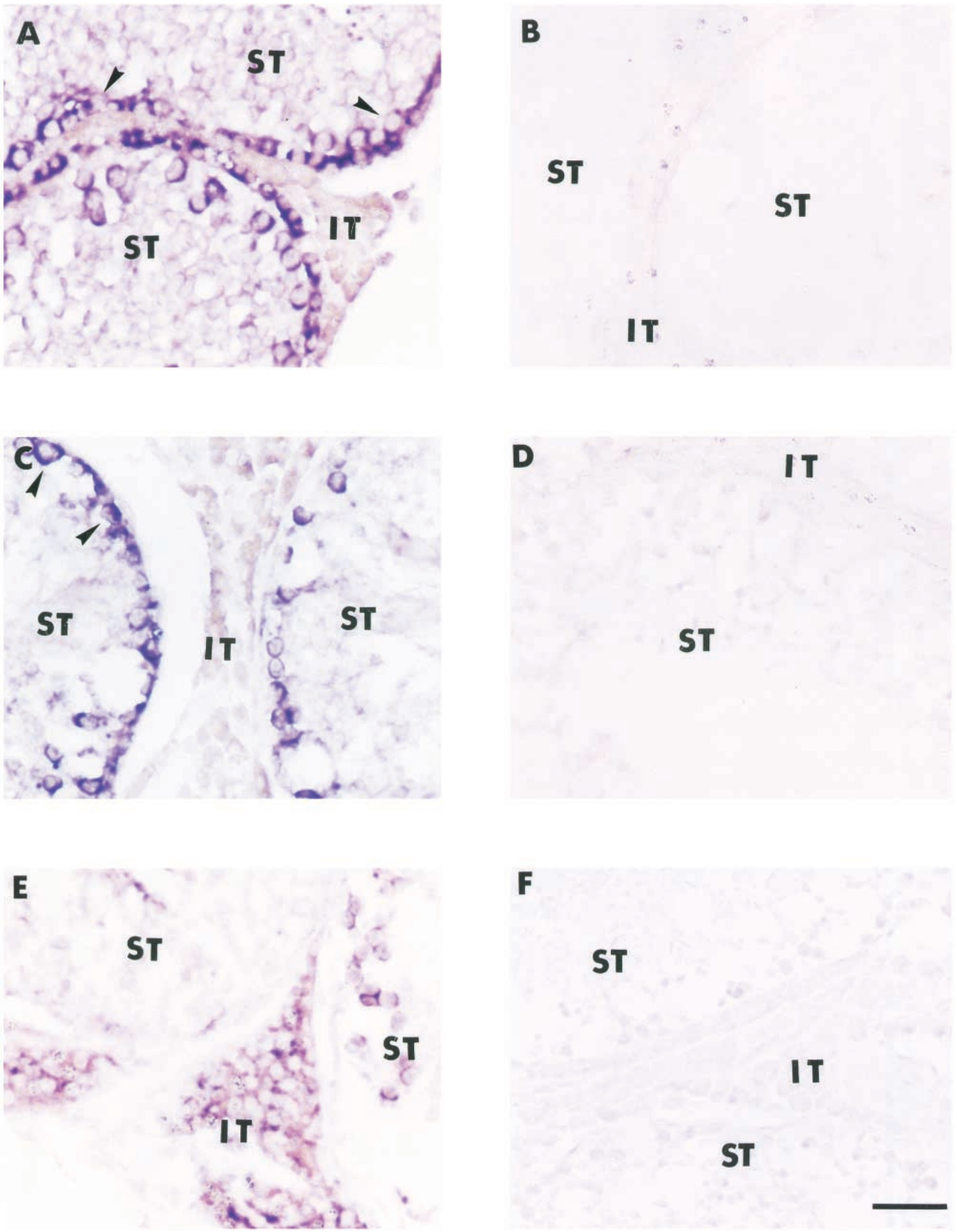
Figure 2. Expression of mRNAs of three porcine inhibin subunits (α, βA, and βB) in testes of Göttingen miniature pig. With the antisense, mRNA signal for inhibin α subunit was observed in peritubular cells, Sertoli cells (arrowheads), and Leydig cells **(A)**. Inhibin βA mRNA signal was observed mainly in Sertoli cells (arrowheads), and in peritubular and Leydig cells **(C)**. Inhibin βB mRNA signal was observed mainly in Leydig cells and in some spermatogonia **(E)**. There were no positive signals with either sense probe **(B, D,** and **F)**. Arrowheads indicate Sertoli cells; ST, seminiferous tubule; IT, interstitial tissue. Bar = 50 μm.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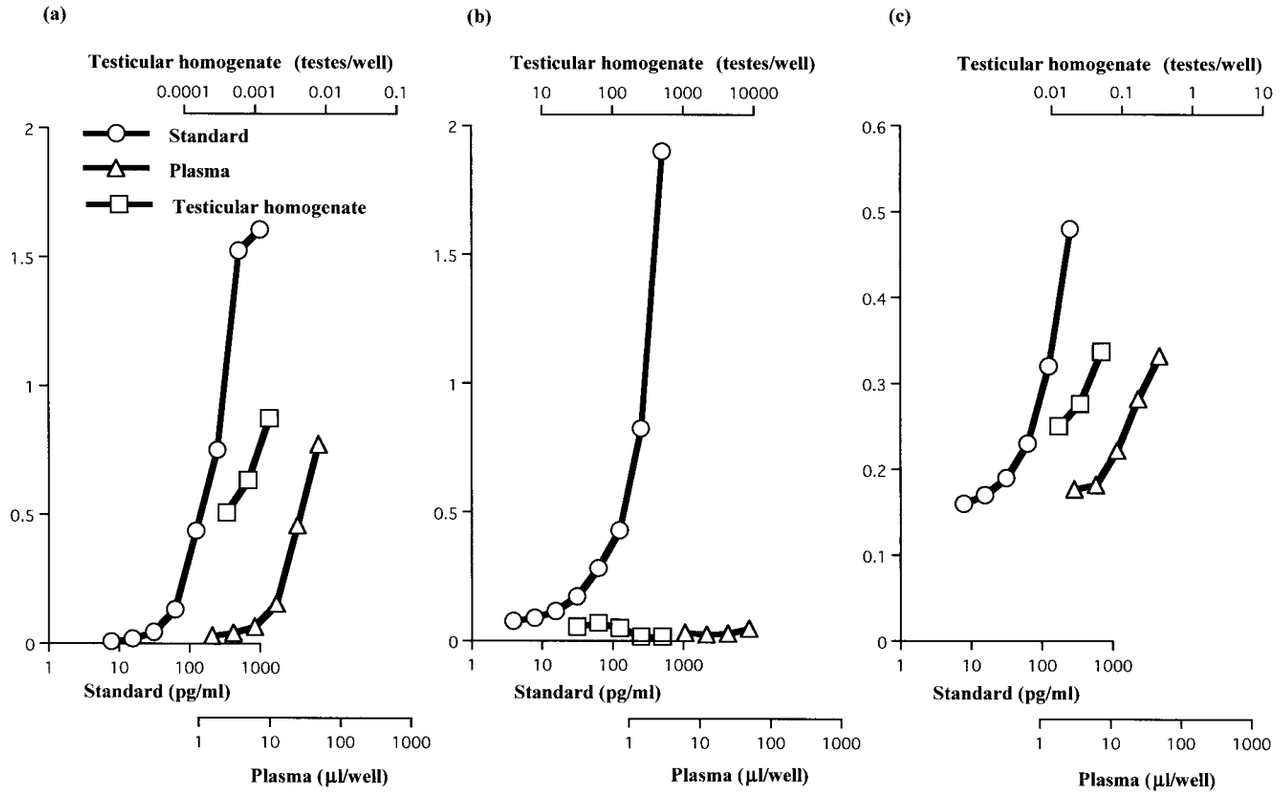

Figure 3. Dose-dependent curves of standards (○), serially diluted peripheral plasma (△), and testicular homogenate (□) of male Göttingen miniature pigs as determined by ELISA. **(a)** Inhibin pro-αC, **(b)** inhibin A, and **(c)** inhibin B.

Concentrations of inhibin A, inhibin B, and inhibin pro-αC in peripheral plasma and testicular homogenates

	Concentration	
	Plasma (pg/mL)	Testicular homogenate (pg/g)
Inhibin A	Undetectable	Undetectable
Inhibin B	126.5 ± 0.8	715.3 ± 0.9
Inhibin pro-αC	169.9 ± 13.9	668.3 ± 13.8

Concentrations of inhibin pro-αC, inhibin A, and inhibin B as determined by ELISA. Values are expressed as means ± SEM for 10 animals.

Kaipia et al 1992). Yamamoto et al (1996) also observed expression of the inhibin βB subunit at different stages of development from spermatogonia to spermatids. Furthermore, Andersson et al (1998) reported that the βB subunit was expressed in germ cells from pachytene spermatocyte to early spermatid stages. The present data also support the hypothesis that inhibin B can be used as a marker of spermatogenesis (Anderson et al, 1998; Pierik et al, 1998; Eckardstein et al, 1999; Foppiani et al, 1999; Ramaswamy et al, 2000).

The present finding that inhibin α subunit protein and its mRNA are present in Sertoli cells suggest that the cells may secrete inhibin α subunit precursor, inhibin pro-αC, and agrees with previous studies in other species (Fujimura et al, 1998; Nagata et al, 1998; Roberts et al, 1989; Kishi et al, 2000; Jin et al, 2001). Precursors of inhibin α subunit have been reported to modulate FSH action (Schneyer et al, 1991) and have been found to play an important role in male reproductive physiology (Tuohimaa et al, 1993; Nagata et al, 2000).

In conclusion, the present results suggest that the major form of inhibin in male GM pigs is inhibin B and that Leydig cells appear to be the main source of this inhibin.

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