Journal of Andrology, Vol. 23, No. 2, March/April 2002
Copyright © American Society of Andrology

The Testis as a Major Source of Circulating Inhibins in the Male Equine Fetus During the Second Half of Gestation

YUMIKO TANAKA,*† HIROYUKI TANIYAMA,‡ NOBUO TSUNODA,§ HIROMI SHINBO,†
NATSUKO NAGAMINE,*†§ YASUO NAMBO,‖ SHUN-ICHI NAGATA,¶ GEN WATANABE,*†
CHANDANA B. HERATH,† NIGEL P. GROOME,# AND KAZUYOSHI TAYA*†

*From the *Department of Basic Veterinary Science, The United Graduate School of Veterinary Science, Gifu University, Gifu, Japan; the †Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Tokyo, Japan; the ‡Rakuno Gakuen University, Hokkaido, Japan; the §Shadai Corporation, Hokkaido, Japan; the ‖Equine Research Institute, Japan Racing Association, Tochigi, Japan; the ¶Laboratory of Racing Chemistry, Tochigi, Japan; and the #School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, United Kingdom.*

ABSTRACT: Immunolocalization of the inhibin (α) and inhibin/activin (βA and βB) subunit proteins in equine fetal testes was investigated to determine the ability of the fetal testis to produce inhibins at 120, 150, 200, and 250 days of gestation. In addition, concentrations of immunoreactive (ir)-inhibin, inhibin pro-αC, and inhibin A in both the maternal and fetal circulation were measured. It was found that plasma concentrations of ir-inhibin, inhibin pro-αC, and inhibin A were much higher ($P < .05$) in the fetal than in the maternal circulation at any stage of gestation examined. Similarly, while fetal testicular homogenate contained increased amounts of inhibins, the inhibins were undetectable in homogenates of maternal ovaries and placentae. At 120 days of gestation, all 3 subunit proteins were localized to the interstitial cells, while the immunoreactivity for the inhibin/activin βB subunit protein was also observed in Sertoli cells. The intensity of immunoreactivity for the 3 subunit proteins in interstitial cells increased as pregnancy advanced to day 200, and, at this stage, immunoreactivity for the inhibin α subunit protein was observed in the fetal testes in a pattern consistent with localization in Sertoli cells. Thus, the inhibin/activin βA subunit protein was confined to interstitial cells during the gestational periods examined. We conclude that equine fetal testes secrete large amounts of inhibins, including dimeric inhibin A and possibly other dimeric forms, such as inhibin B and activins, into the fetal circulation. These results suggest that these proteins may play some important roles in the development of fetal testes during gestation.

Key words: Horse, fetal tests, inhibin A, inhibin pro-αC, Sertoli cells, interstitial cells.

J Androl 2002;23:229–236

Inhibins belonging to the transforming growth factor β (TGF-β) superfamily (Mason et al, 1986; Stewart et al, 1986; DePaolo et al, 1991; Dye et al, 1992; Halvorson and DeCherney, 1996) were initially discovered as gonadal peptides that preferentially inhibit follicle-stimulating hormone (FSH) secretion from the pituitary gland (Burger and Igarashi, 1988). Inhibin consists of an α subunit linked by a disulfide bridge to one of the 2 highly homologous β subunits (βA and βB) to form inhibin A (α and βA) or inhibin B (α and βB). Inhibin subunits have been detected in fetal testes in humans (Ravinovici et al, 1991; Majdic et al, 1997), monkeys (Ravinovici et al, 1991), rats (Shaha et al, 1989; Majdic et al, 1997), and sheep (Thomas et al, 1995; Jarred et al, 1999). The cellular localization of inhibin subunits in the testis varies among species and during fetal development. Previous reports suggested that these proteins may play some important roles in spermatogonial development (van Dissel-Emiliani et al, 1989; Mather et al, 1990), Sertoli cell proliferation (Lin et al, 1989; Boitani et al, 1995), and steroid biosynthesis (Hsueh et al, 1987; Lin et al, 1989; Mauduit et al, 1991; de Winter et al, 1993; Majdic et al, 1997). The cellular localization of inhibin subunits has been investigated in both adult female and adult male gonads in the horse (Nagamine et al, 1998; Nagata et al, 1998a,b). In addition, other studies, including those from our laboratory, have reported the localization of the inhibin α subunit messenger ribonucleic acid (mRNA) or protein in the equine fetal gonads (Nambo et al, 1996; Yamanouchi et al, 1997). However, the immunolocalization of all 3 subunit proteins, such as the inhibin α and inhibin/activin βA and βB in the gonads, together with plasma and testicular concentrations of immunoreactive (ir)-inhibin, inhibin pro-αC, and dimeric inhibin A, has not been doc-

Supported by a grant-in-aid from the Equine Research Institute of the Japan Racing Association.

Correspondence to: Kazuyoshi Taya, DVM, PhD, Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan (e-mail: taya@cc.tuat.ac.jp).

Received for publication March 15, 2001; accepted for publication September 20, 2001.

umented for the equine fetus. The equine fetal gonads of both sexes have attracted much interest because of a remarkable enlargement of gonadal size resulting from an increased number of interstitial cells. It is also known that the size of the equine fetal gonads during the second half of gestation is larger than that of the maternal ovaries (Cole et al, 1933). Although the mechanism and physiological significance of this enlargement of fetal gonads are not clear, a previous study showed that gonadectomy in the equine fetus affected fetal development (Pashen and Allen, 1979). The aim of the present study, therefore, is to use recently developed enzyme-linked immunosorbent assay (ELISA) techniques and also immunohistochemistry to investigate the ontogeny of inhibin production during the period of accelerated growth of the equine fetal testes.

Materials and Methods

Animals and Blood Samples

Sixteen pregnant Thoroughbred and Anglo-Arab mares with known insemination dates were sacrificed by an overdose of a mixture of sodium thiopental (Ravonal, Tanabe Pharmaceutical Co Ltd, Osaka, Japan) and suxamethonium chloride (Succine, Yamanouchi Pharmaceutical Co Ltd, Osaka, Japan) between 120 and 250 days of gestation (full term = 340 days). The day of mating was designated as day 0 of gestation. Nine of the mares carried male fetuses at gestational days 120 (n = 2), 150 (n = 3), 200 (n = 2), and 250 (n = 2). These 9 mares and their male fetuses were used in the present study. The maternal blood samples were collected by venipuncture from the jugular vein, and the fetal blood samples were collected by cardiac puncture immediately after euthanasia. All blood samples were collected into plastic tubes containing heparin (20 IU/mL blood) as anticoagulant to prevent clotting. The samples were stored in ice and centrifuged at 1700 × *g* for 15 minutes at 4°C immediately after completing the experiment. The resulting plasma was harvested and stored at −20°C until assayed for inhibins. All procedures were carried out in accordance with the guidelines established by the Rakuno Gakuen University (Hokkaido, Japan) for use of laboratory animals.

Fetal Testicular Samples

One testis from each fetus was removed, fixed in freshly prepared 4% (wt/vol) paraformaldehyde (Sigma Chemical Co, St Louis, Mo) in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and embedded in paraffin. For immunohistochemical examination, 5-μm-thick tissue sections from the testis were prepared. The other testis was homogenized (1 g of testis in 2 mL 0.01 M PBS, pH 7.4). The homogenized testicular samples were centrifuged at 20 000 × *g* for 15 minutes at 4°C, and the supernatant was removed and stored at −80°C until assayed for inhibins.

Radioimmunoassay

Concentrations of ir-inhibin in plasma and testicular homogenates were measured by a double-antibody radioimmunoassay, as described previously (Hamada et al, 1989). Purified bovine 32-kd inhibin was used as a standard. The same material was labeled with ^{125}I according to the chloramine-T method. The antiserum used in this assay was raised against bovine 32-kd inhibin (TNDH-1) in a castrated male rabbit. The assay system does not distinguish dimeric inhibin from α subunit monomer. We have validated the assay system for equine plasma as well as ovarian and testicular homogenates, as described previously (Nambo et al, 1996). In that study, we have reported the parallelism tested for ir-inhibin in equine follicular fluid, pregnant and cyclic mare plasma, and homogenates of placenta, fetal ovaries, newborn foal ovaries, and newborn foal testes. The results were expressed in terms of 32-kd bovine inhibin. The sensitivity of the assay was 7.8 pg/tube (78 pg/mL). Intra- and interassay coefficients of variation were 8.0% and 16.2%, respectively.

Enzyme-Linked Immunosorbent Assay

Concentrations of inhibin pro-αC in plasma and testicular homogenates were measured using a 2-site ELISA kit (Serotec, Oxford, United Kingdom) designed for measurement of human inhibin pro-αC. In the assay, 2 monoclonal antibodies directed against the pro- and αC regions were used (Groome et al, 1995). Serial dilutions of pooled equine plasma and testicular homogenates were assayed to test for parallelism.

Concentrations of inhibin A in plasma and testicular homogenates were measured using a 2-site ELISA kit. The preparation of a new monoclonal antibody to the α subunit of cow inhibin (PPG1/14/6), together with an E4 monoclonal antibody to the inhibin/activin βA subunit, has produced an ELISA able to measure inhibin A in sheep (Knight et al, 1998), cows (Bleach et al, 2001), and horses, each with a similar sensitivity to the human inhibin A ELISA.

Immunohistochemistry

The paraffin-embedded testes sectioned at 5 μm were placed on glass slides coated with 3-aminopropyltriethoxysilane. The sections were deparaffinized with xylene, passed through a graded series of alcohol, washed in PBS, and prepared for immunohistochemical staining. Antigenic sites were exposed by heating to 100°C in a microwave for 15 minutes and allowed to cool for 20 minutes. The sections were then treated with 3% H_2O_2 in methanol at room temperature for 15 minutes and incubated with Block Ace (Dainippon Pharmaceutical Co Ltd, Osaka, Japan) for 1 hour at 37°C to block nonspecific binding. The sections were incubated with primary antibodies overnight at 4°C.

To detect inhibin α subunit, rabbit polyclonal antibody against [Tyr30] porcine inhibin α-chain (120)-NH_2 ([Tyr30] porcine inhibin α-chain (120)-NH_2 was kindly provided by Dr N. Ling, Neurocrine Inc, San Diego, Calif) was used at a dilution of 1:1000. To detect inhibin/activin βA and βB subunits, monoclonal antibodies raised against the synthetic peptides corresponding to amino acid sequences of 82–114 of human βA subunit (E4) or βB subunit (C5) were used at a dilution of 1:3000 (Nagamine et al, 1998). After washing with PBS, the sections were incubated for 1 hour at 37°C with 0.5% (vol/vol in PBS) biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vectastain ABC Kit Elite, Vector Labs, Burlingame, Calif) for the α subunit and 0.5% (vol/vol) biotinylated goat anti-mouse IgG (Vectastain

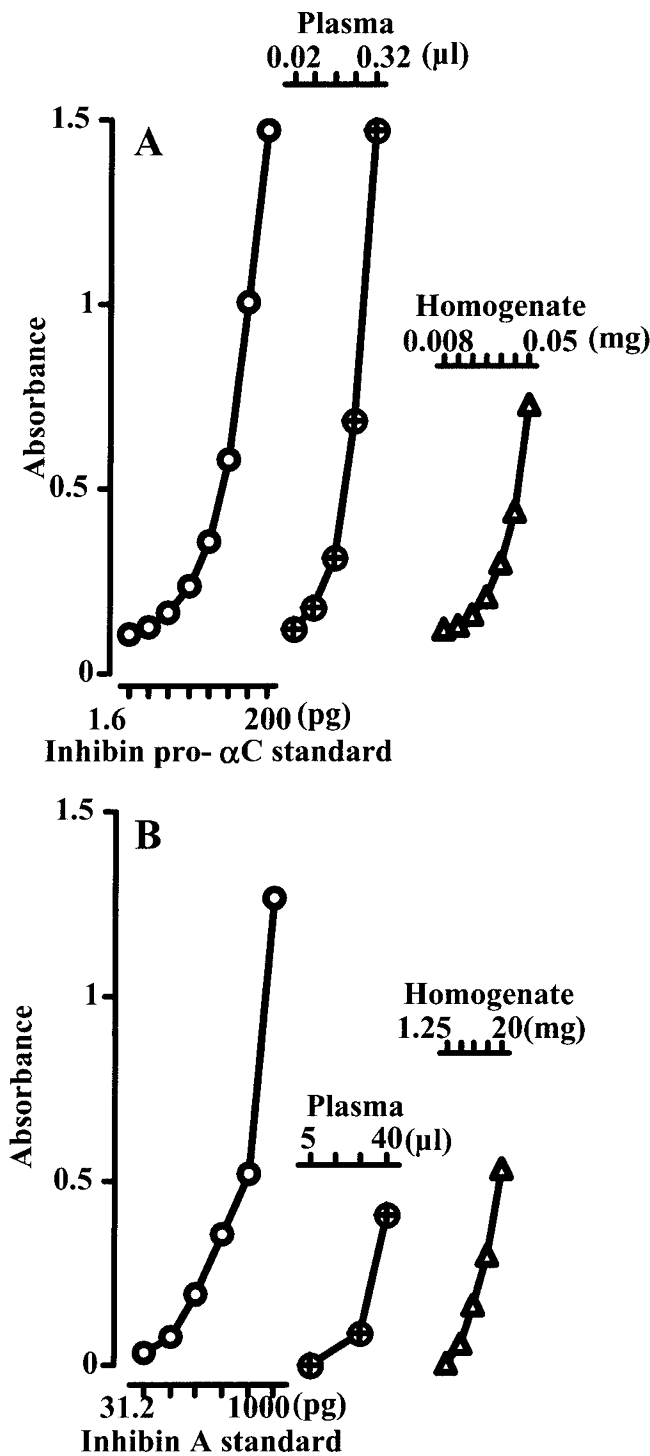


Figure 1. Validation of the enzyme-linked immunosorbent assay (ELISA) systems for equine samples. **(A)** Different dilutions of equine fetal plasma (⊕) and equine testicular homogenates (Δ—Δ) produced dose-response curves, which were parallel to the standard curves (○) produced with human inhibin pro-αC **(A)** and bovine 32-kd inhibin **(B)**.

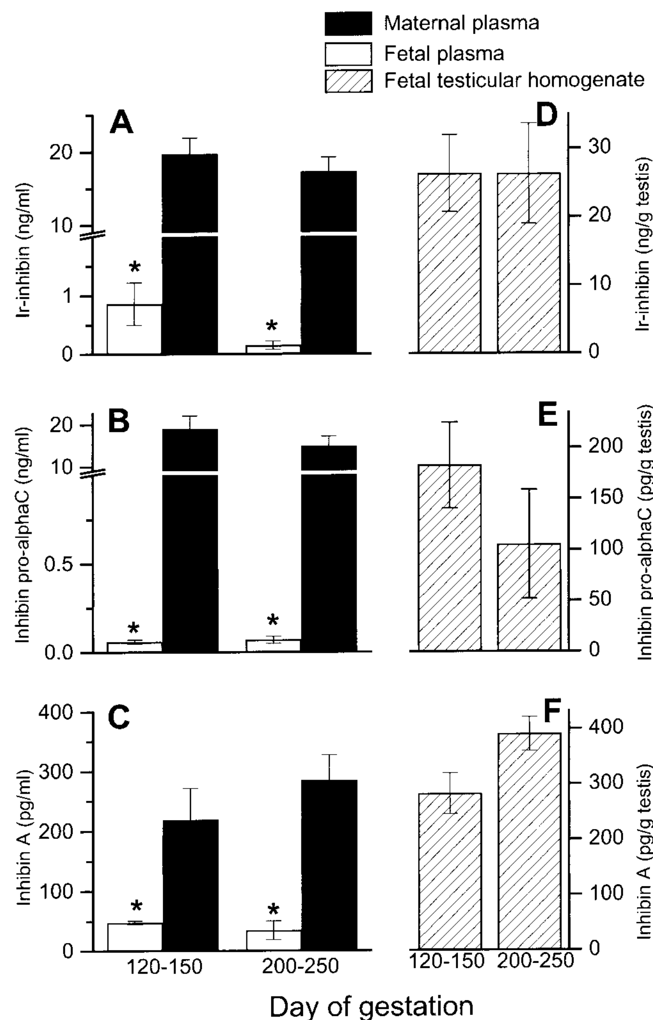
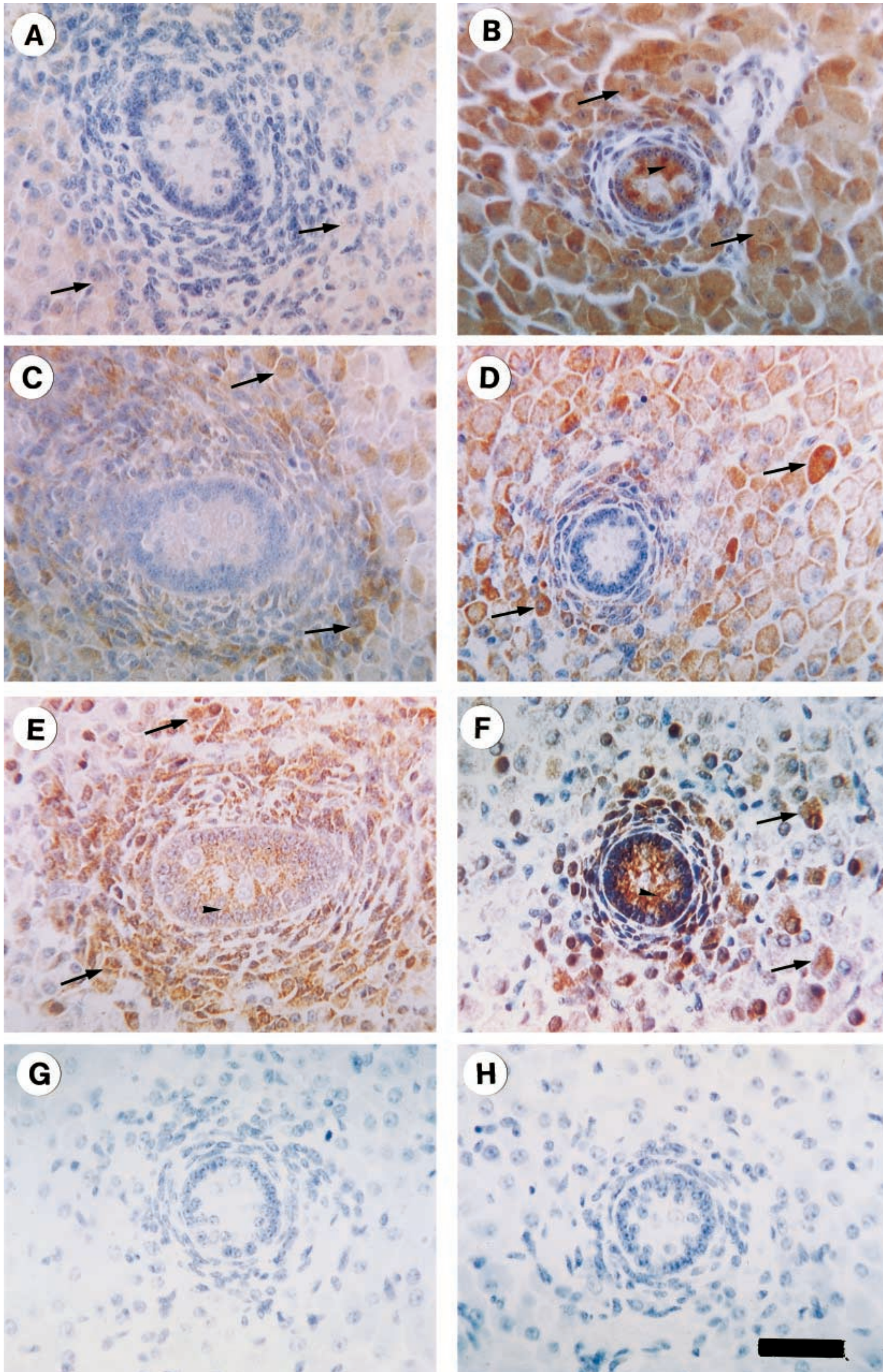


Figure 2. Plasma concentrations of immunoreactive (ir)-inhibin **(A)**, inhibin pro-αC **(B)**, and inhibin A **(C)** in fetuses (solid bars) and dams (open bars) at 120–150 days (n = 5) and 200–250 days (n = 4) of gestation. Plasma concentrations of ir-inhibin **(D)**, inhibin pro-αC **(E)**, and inhibin A **(F)** in fetal testicular homogenates at 120–150 days (n = 5) and 200–250 days (n = 4) of gestation. *$P < .05$, significance of differences between the maternal and fetal hormone concentrations. Vertical bars indicate standard error of the mean.

ABC Kit Elite) for the βA and βB subunits, and then were washed in PBS. All sections were incubated with 2% (vol/vol in PBS) avidin-biotin complex (Vectastain ABC Kit Elite) for 30 minutes at 37°C. The primary antibody bound to the sections was visualized by treating with 0.05% (wt/vol in PBS) 3,3′-diaminobenzidine tetrachloride (Sigma) containing 0.01% H_2O_2. Specificity of the antibodies was examined using normal rabbit serum (NRS) or normal mouse serum (NMS), instead of primary antibody, using a 1:1000 dilution. All tissue sections were counterstained with hematoxylin for 15 seconds, dehydrated, cleared, and mounted.

Statistical Analysis

One-way analysis of variance was carried out, and the significance between means was determined by the Student's *t* test.


A
B
C
D
E
F
G
H

Data are presented as means ± SEM. A P-value less than .05 was considered to be significant.

Results

Characterization of the Inhibin Pro-αC and Inhibin A ELISA Systems

Dose-response curves of serially diluted plasma and testicular homogenate obtained from fetuses at 200 days of gestation produced suppression curves, which were parallel to the standard curves produced with human inhibin pro-αC (Figure 1A) and inhibin A (Figure 1B). This indicated that it was possible to measure concentrations of inhibin pro-αC and inhibin A in both fetal plasma and testicular homogenate using these ELISA systems.

Plasma Concentrations of ir-Inhibin, Inhibin Pro-αC, and Inhibin A in Fetuses and Dams

Concentrations of ir-inhibin, inhibin pro-αC, and inhibin A in peripheral plasma of fetuses and dams were not different between 120 (n = 2) and 150 (n = 3) days or between 200 (n = 2) and 250 (n = 2) days of gestation. Therefore, data from the groups at 120 and 150 days and at 200 and 250 days of gestation were combined to represent plasma concentrations at 120–150 days (n = 5) and at 200–250 days (n = 4) of gestation, respectively. At both 120–150 and 200–250 days of gestation, plasma concentrations of ir-inhibin (Figure 2A), inhibin pro-αC (Figure 2B), and inhibin A (Figure 2C) were significantly ($P < .05$) higher in the male fetuses than in the dams. Plasma concentrations of these inhibins were not different between 120–150 and 200–250 days of gestation in either the fetuses or the dams.

Concentrations of ir-Inhibin, Inhibin Pro-αC, and Inhibin A in Fetal Testicular Homogenates

Elevated concentrations of ir-inhibin (Figure 2D), inhibin pro-αC (Figure 2E), and inhibin A (Figure 2F) were detected in testicular homogenates of fetuses at both 120–150 (n = 5) and 200–250 (n = 4) days of gestation. In contrast, these inhibin forms were undetectable in both maternal ovaries and placentae obtained at these times (data not shown).

Immunohistochemistry

Since plasma concentrations of all inhibin forms investigated in this study were not different in the fetuses between 120 and 150 days and 200 and 250 days of gestation, Figure 3 shows immunoreactivity for the inhibin α and for the inhibin/activin βA and βB subunit proteins in the equine fetal testes obtained from fetuses at 120 and 200 days of gestation only.

Equine fetal testicular tissue sections stained with specific antibody were positive for the inhibin α subunit in the interstitial cells (arrows) but not in the Sertoli cells at 120 days of gestation (Figure 3A). As pregnancy advanced, the interstitial cells (arrows) were intensely stained for the inhibin α subunit at 200 days of gestation (Figure 3B). In particular, the interstitial cells located closely around seminiferous tubules stained prominently. On the other hand, at this time, Sertoli cells (arrowhead) also stained positively for the inhibin α subunit (Figure 3B). It was also found that the intensity of immunostaining for the inhibin α subunit protein in both interstitial and Sertoli cells was highest at 200 days of gestation compared with all other stages examined in this study.

Immunoreactivity for the inhibin/activin βA subunit protein was somewhat similar to that observed for the inhibin α subunit. Thus, the interstitial cells (arrows) stained positive for the inhibin/activin βA subunit protein at 120 days of gestation (Figure 3C), and the intensity increased as pregnancy advanced (Figure 3D). However, it was apparent that the Sertoli cells were not stained for the inhibin/activin βA subunit protein at any of the stages examined.

In contrast, immunostaining for the inhibin/activin βB subunit protein was observed in both interstitial cells (arrows) and Sertoli cells (arrowhead) at 120 days of gestation (Figure 3E). Similar to the α subunit-staining specificity, the intensity of the inhibin/activin βB subunit immunostaining in both cell types increased at 200 days of gestation, though the number of cells stained in the interstitial tissue appeared to have become fewer (Figure 3F).

Testicular tissue sections obtained from fetuses at 200 days of gestation and incubated with NRS (Figure 3G) or NMS (Figure 3H) instead of primary antibody did not show immunostaining reaction in either the interstitial or the Sertoli cells. The nuclei of different cell types that

←

Figure 3. Photomicrographs of an equine fetal testis showing cross-sections of interstitial tissue and a seminiferous tubule as well as inhibin subunit-staining specificity. Tissue sections stained with specific antibodies were strongly positive for the inhibin α **(A)** and inhibin/activin βA subunit proteins **(C)** in the interstitial cells (arrows) and inhibin/activin βB subunit protein **(E)** in both the interstitial cells (arrows) and Sertoli cells (arrowhead) at 120 days of gestation. Tissue sections stained with specific antibodies were strongly positive for the inhibin α **(B)** and inhibin/activin βB subunit proteins **(F)** in both the interstitial cells (arrows) and Sertoli cells (arrowhead) and inhibin/activin βA subunit protein **(D)** in the interstitial cells (arrows) at 200 days of gestation. Sections incubated with normal rabbit serum (NRS) **(G)** or normal mouse serum (NMS) **(H)** instead of primary antibody did not show immunostaining reaction. All sections were counterstained with hematoxylin for 15 seconds. The nuclei of cells that stained blue with hematoxylin can be seen in all sections **(A through H)**. Scale bar = 50 μm.

stained blue with hematoxylin can be seen in all sections (Figure 3A through H).

Discussion

The present study clearly indicates that equine fetal testes contain large amounts of inhibin pro-αC and inhibin A. Similarly, the present study shows that concentrations of these inhibin forms in the fetal circulation are several-fold higher than those of the maternal circulation. In line with elevated plasma inhibin levels in the fetuses, we found that the inhibin (α) and inhibin/activin (βA and βB) subunit proteins were clearly localized to the relatively enlarged interstitial cells of the equine fetal testes. Thus, the present results strongly suggest that equine fetal testes secrete large amounts of dimeric inhibin A into the fetal circulation. On the basis of immunohistochemical results, it is also equally possible that equine fetal testes have the capacity to secrete other forms of dimeric hormones such as inhibin B and activins.

It has been demonstrated that the immunolocalization of the inhibin α subunit protein coincides with the cellular expression of the inhibin α subunit mRNA in the equine fetal testes (Yamanouchi et al, 1997). The localization of the inhibin α subunit in both the Leydig and Sertoli cells at 200 days of gestation is similar to that of the adult stallion (Nagata et al, 1998a). In the Nagata et al (1998a) study, the inhibin α subunit stained both the Leydig and the Sertoli cells in the 5-year-old adult stallion. Thus, it appears that, similar to the adult testis, equine fetal testes have the capacity to secrete inhibin α subunit protein at 200 days of gestation. Furthermore, previous studies have shown that the inhibin or inhibin/activin subunit proteins are immunolocalized in the fetal testes of various other mammals. In the fetal rat testes, immunostaining for the inhibin α subunit protein is detectable 2 days earlier than that of the inhibin/activin βB subunit protein (Shaha et al, 1989; Majdic et al, 1997; Noguchi et al, 1997). In the present study, the immunostaining pattern of the inhibin α and inhibin/activin βB subunit proteins was almost similar. As pregnancy advanced, the immunostaining reaction appeared more intense in the interstitial cells than in the Sertoli cells. In the human fetal testis at 16 weeks of gestation, inhibin α and inhibin/activin βB subunit proteins are detectable in both the Sertoli and interstitial cells, and the immunostaining reaction becomes more intense in the Sertoli than in the interstitial cells at 24 weeks of gestation (Majdic et al, 1997). Conversely, it has been reported that the amount of inhibin/activin βA subunit protein present in rat and human fetal testes is generally very low (Shaha et al, 1989; Ravinovici et al, 1991; Majdic et al, 1997). On the other hand, there are species-specific differences in the localization of the inhibin and inhibin/activin subunit proteins in the fetal testes. For example, in the ovine fetal testes, the immunostaining for the α, βA, and βB subunit proteins can be detected in both the Sertoli and interstitial cells until the mid-gestational period (Jarred et al, 1999). Thereafter, the presence of the α subunit protein in the interstitial cells is no longer detectable, while the immunointensity of the βA and βB subunit proteins is reduced. However, the localization of all 3 subunit proteins in the Sertoli cells is apparently not changed (Jarred et al, 1999).

In the present study, the immunolocalization of the βB subunit protein, but not the βA subunit protein, was confined to both the Sertoli and interstitial cells of the equine fetal testes, indicating that in addition to the interstitial cells, Sertoli cells also have the capacity to secrete inhibin B and/or activin B into the fetal circulation. In contrast to the present findings, previous studies have reported that no immunostaining reaction can be detected for the βA subunit protein in the fetal testis of rats (Shaha et al, 1989; Majdic et al, 1997), rhesus monkeys (Rabinovici et al, 1991), and humans (Rabinovici et al, 1991; Majdic et al, 1997). Expression of the βA subunit mRNA in the mature rat testis has been reported to be lower than those of the inhibin α and βB subunits, and the βA subunit expression level declined rapidly with maturation (Meunier et al, 1988). In addition, it was reported that circulating inhibin A concentrations are undetectable in adult men and that a significant correlation exists between plasma concentrations of FSH and inhibin B (Illingworth et al, 1996). These results suggest that inhibin B and/or inhibin βB subunit-related substances are possible candidates to play physiologically important roles in male reproduction. In contrast, in equine male fetuses, inhibin A was also thought to be an important dimeric protein. In the present study, the fetal testes contained large amounts of inhibins, and the circulating concentrations in the fetuses were much higher than those in the dams at any stage of gestation examined. Additionally, we found that inhibins were undetectable in ovaries and placentae of pregnant mares. These results indicate that fetal testes were the source of inhibins in the fetal circulation. The immunostaining for all 3 subunit proteins appeared to be most intense at 200 days of gestation. This result is probably associated with an increased size of the equine fetal testes, which occurs during the second half of equine gestation. Previous studies have shown that this massive increase in the size of fetal gonads results from an increased proliferation and hypertrophy of interstitial cells (Hay and Allen, 1975; Tsunoda et al, 1996) and that these cells had the ultrastructural characteristics of steroid-secreting cells (Gonzalez-Angulo et al, 1975; Hay and Allen, 1975). Although the mechanism of this enlargement is not known, large amounts of inhibins that are present in both the testes and fetal circulation may possibly be involved in these

hypertrophic changes of the gonads. In addition, the equine fetal gonads have also been identified as a source of dehydroepiandrosterone (DHEA), which is one of the precursor forms of maternal estrogen during pregnancy (Hay and Allen, 1975). This led researchers to consider the equine fetal gonad an important organ in steroid biosynthesis. Furthermore, the present results clearly indicate that enlarged fetal gonads have the ability to produce large amounts of inhibin and/or activins. Further studies are required to determine which forms of inhibins/activins are important and what physiological roles they play during equine gestation. Some investigators have hypothesized local effects of inhibin and activin in the testes. Activin stimulated the proliferation of Sertoli cells and spermatogonia in vitro (Mather et al, 1990; de Winter et al, 1993; Boitani et al, 1995), while inhibin decreased the number of differentiating spermatogonia when injected locally into the testis of adult hamsters (van Dissel-Emiliani et al, 1989). Furthermore, activin inhibits human chorionic gonadotropin (hCG)-stimulated testosterone production, and inhibin has been found to be without effect. However, inhibin reverses the inhibitory action of activin in both mature and immature Leydig cells (Lin et al, 1989; Mauduit et al, 1991) and facilitates luteinizing hormone (LH) action in immature Leydig cells (Mauduit et al, 1991). In addition, activin inhibits FSH-stimulated aromatase activity, androgen receptor mRNA expression, and androgen binding in immature Sertoli cells (de Winter et al, 1993). In light of these reports, our results suggest that the equine fetal testis can also secrete inhibins and activins and that these dimeric proteins may be important for the regulation of testicular cell differentiation and steroidogenesis in equine fetal testes.

We conclude that equine fetal testes secrete large amounts of inhibins into the fetal circulation. In addition, the presence of all 3 subunit proteins suggests that equine fetal testes have the capacity to secrete other forms of dimeric proteins such as inhibin B and activins. These results together with data reported in the literature suggest that inhibins may have physiological roles as regulators of development of testicular functions in equine fetuses.

Acknowledgment

We are grateful to Dr N. Ling (Neurocrine Inc, San Diego, Calif) for providing [Tyr^{30}]-porcine inhibin α(1–30).

References

Bleach EC, Glencross RG, Feist SA, Groome NP, Knight PG. Plasma inhibin A in heifers: relationship with follicle dynamics, gonadotropins, and steroids during the estrous cycle and after treatment with bovine follicular fluid. *Biol Reprod.* 2001;64:743–752.

Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. *Endocrinology.* 1995;136:5438–5444.

Burger HG, Igarashi M. Inhibin: definition and nomenclature, including related substances. *Endocrinology.* 1988;122:1701–1702.

Cole HH, Hart GH, Lyons WR, Catchpole HR. The development and hormonal content of fetal horse gonads. *Anat Rec.* 1933;56:275–293.

DePaolo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N. Follistatin and activin: a potential intrinsic regulatory system within diverse tissues. *Proc Soc Exp Biol Med.* 1991;198:500–512.

De Winter JP, Vanderstichele HM, Timmerman MA, Blok LJ, Themmen AP, de Jong FH. Activin is produced by rat Sertoli cells in vitro and can act as an autocrine regulator of Sertoli cell function. *Endocrinology.* 1993;132:975–982.

Dye RB, Rabinovici J, Jaffe RB. Inhibin and activin in reproductive biology. *Obstet Gynecol Surv.* 1992;47:173–185.

Gonzalez-Angulo A, Hernandez-Jauregui P, Martinez-Zedilo G. Fine structure of the gonads of the horse and its functional implications. *J Reprod Fertil.* 1975;23(suppl):563–567.

Groome NP, Illingworth PJ, O'Brien M, Priddle J, Weaver K, McNeilly AS. Quantification of inhibin pro–αC-containing forms in human serum by a new ultrasensitive two-site enzyme-linked immunosorbent assay. *J Clin Endocrinol Metab.* 1995;80:2926–2932.

Halvorson LM, DeCherney AH. Inhibin, activin, and follistatin in reproductive medicine. *Fertil Steril.* 1996;65:459–469.

Hamada T, Watanabe G, Kokuho T, Taya K, Sasamoto S, Hasegawa Y, Miyamoto K, Igarashi M. Radioimmunoassay of inhibin in various mammals. *J Endocrinol.* 1989;122:697–704.

Hay MF, Allen WR. An ultrastructural and histochemical study of the interstitial cells in the gonads of the fetal horse. *J Reprod Fertil.* 1975; 23(suppl):557–561.

Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW, Vale W. Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci USA.* 1987;84:5082–5086.

Illingworth PJ, Groome NP, Byrd W, Rainey WE, McNeilly AS, Mather JP, Bremner WJ. Inhibin-B: a likely candidate for the physiologically important form of inhibin in men. *J Clin Endocrinol Metab.* 1996; 81:1321–1325.

Jarred RA, Cancilla B, Richards M, Groome NP, McNatty KP, Risbridger GP. Differential localization of inhibin subunit proteins in the ovine testis during fetal gonadal development. *Endocrinology.* 1999;140: 979–986.

Knight PG, Feist SA, Tannetta DS, Bleach EC, Fowler PA, O'Brien M, Groome NP. Measurement of inhibin-A (α A dimer) during the oestrous cycle, after manipulation of ovarian activity and during pregnancy in ewes. *J Reprod Fertil.* 1998;113:159–166.

Lin T, Calkins JK, Morris PL, Vale W, Bardin CW. Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology.* 1989;125:2134–2140.

Majdic G, McNeilly AS, Sharpe RM, Evans LR, Groome NP, Saunders PT. Testicular expression of inhibin and activin subunits and follistatin in the rat and human fetus and neonate and during postnatal development in the rat. *Endocrinology.* 1997;138:2136–2147.

Mason AJ, Niall HD, Seeburg PH. Structure of two human ovarian inhibins. *Biochem Biophys Res Commun.* 1986;135:957–964.

Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM. Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology.* 1990;127:3206–3214.

Mauduit C, Chauvin MA, de Peretti E, Morera AM, Benahmed M. Effect of activin A on dehydroepiandrosterone and testosterone secretion by primary immature porcine Leydig cells. *Biol Reprod.* 1991;45:101–109.

Meunier H, Rivier C, Evans RM, Vale W. Gonadal and extragonadal expression of inhibin alpha, beta A, and beta B subunits in various

tissues predicts diverse functions. *Proc Natl Acad Sci USA*. 1988;85: 247–251.

Nagamine N, Nambo Y, Nagata S, et al. Inhibin secretion in the mare: localization of inhibin α, βA, and βB subunits in the ovary. *Biol Reprod.* 1998;59:1392–1398.

Nagata S, Miyake YI, Nambo Y, et al. Inhibin secretion in the stallion. *Equine Vet J.* 1998a;30:98–103.

Nagata S, Tsunoda N, Nagamine N, Tanaka Y, Taniyama H, Nambo Y, Watanabe G, Taya K. Testicular inhibin in the stallion: cellular source and seasonal changes in its secretion. *Biol Reprod.* 1998b;59:62–68.

Nambo Y, Nagata S, Oikawa M, et al. High concentrations of immunoreactive inhibin in the plasma of mares and fetal gonads during the second half of pregnancy. *Reprod Fertil Dev*. 1996;8:1137–1145.

Noguchi J, Hikono H, Sato S, Watanabe G, Taya K, Sasamoto S, Hasegawa Y. Ontogeny of inhibin secretion in the rat testis: secretion of inhibin-related proteins from fetal Leydig cells and of bioactive inhibin from Sertoli cells. *J Endocrinol.* 1997;155:27–34.

Pashen RL, Allen WR. The role of the fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. *J Reprod Fertil.* 1979;27(suppl):499–509.

Rabinovici J, Goldsmith PC, Roberts VJ, Vaughan J, Vale W, Jaffe RB. Localization and secretion of inhibin/activin subunits in the human and subhuman primate fetal gonads. *J Clin Endocrinol Metab.* 1991; 73:1141–1149.

Shaha C, Morris PL, Chen CL, Vale W, Bardin CW. Immunostainable inhibin subunits are in multiple types of testicular cells. *Endocrinology.* 1989;125:1941–1950.

Stewart AG, Milborrow HM, Ring JM, Crowther CE, Forage RG. Human inhibin genes. Genomic characterisation and sequencing. *FEBS Lett.* 1986;206:329–334.

Thomas GB, Davidson EJ, Engelhardt H, Baird DT, McNeilly AS, Brooks AN. Expression of mRNA and immunocytochemical localization of inhibin alpha- and inhibin beta A-subunits in the fetal sheep testis. *J Endocrinol.* 1995;145:35–42.

Tsunoda N, Machida N, Nagata S, et al. A morphometric study of interstitial cells in equine fetal gonads. *J Reprod Dev*. 1996;42:j91–j95.

Van Dissel-Emiliani FM, Grootenhuis AJ, de Jong FH, de Rooij DG. Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. *Endocrinology.* 1989;125:1899–1903.

Yamanouchi K, Hirasawa K, Hondo E, et al. Expression and cellular localization of inhibin alpha-subunit mRNA in equine fetal gonads. *J Vet Med Sci.* 1997;59:569–573.