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The expression and localization of inhibin isotypes in mouse testis during postnatal development

Yujin Kim[1], Joong-Sun Kim[1], Myoung-Sub Song[1], Heung-Sik Seo[1], Jong Choon Kim[2], Chun-Sik Bae[3], Seungjoon Kim[4], Taekyun Shin[5], Sung-Ho Kim[1], Changjong Moon[1,*]

Departments of [1]Veterinary Anatomy, [2]Veterinary Toxicology, [3]Veterinary Surgery, College of Veterinary Medicine and Veterinary Medical Research Center, Chonnam National University, Gwangju 500-757, Korea
[4]Department of Veterinary Obstetrics, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea
[5]Department of Veterinary Anatomy, College of Veterinary Medicine and Applied Radiological Science Research Institute, Cheju National University, Jeju 690-756, Korea

Inhibin, which is important for normal gonadal function, acts on the pituitary gonadotropins to suppress follicle-stimulating hormone (FSH) secretion. The level and cellular localization of the inhibin isotypes, α, β_A and β_B, in the testis of mice were examined during postnatal development in order to determine if inhibin expression is related to testicular maturation. Mouse testes were sampled on postnatal days (PNDs) 1, 3, 6, 18, 48 and 120, and analyzed by Western blotting and immunofluorescence. Western blot analysis showed very low levels of inhibin α, β_A and β_B expression in the testes at days 1 to 6 after birth. The levels then increased gradually from PND 18 to 48-120, and there were significant peaks at PND 48. Inhibin α, β_A and β_B were detected in testicular cells during postnatal development using immunohistochemistry. The immunoreactivity of inhibin α was rarely observed in testicular cells during PND 1 to 6, or in the cytoplasmic process of Sertoli cells surrounding the germ cells and interstitial cells during PND 18 to 120. Inhibin β_A and β_B immunoreactivity was rarely observed in the testis from PND 1 to 6. On the other hand, it was observed in some spermatogonial cells, as well as in the interstitial space between PND 48 and PND 120. We conclude that the expression of inhibin isotypes increases progressively in the testis of mice with increasing postnatal age, suggesting that inhibin is associated with a negative feedback signal for FSH in testicular maturation.

Keywords: inhibin, mouse, postnatal development, Sertoli cell, testis

*Corresponding author
Tel: +82-62-530-2838; Fax: +82-62-530-2841
E-mail: moonc@chonnam.ac.kr

Introduction

Inhibin is a glycoprotein hormone that is produced principally by the gonads. It is a disulfide linked dimer of two different subunits, a common α isotype and a β_A isotype forming inhibin A subunit or a β_B isotype forming inhibin B subunit [21]. Although five distinct β isotypes have been isolated, which are termed β_A to β_E, only the biological activity of β_A and β_B has been demonstrated [11]. Inhibin belongs to the transforming growth factor β superfamily of growth and differentiation factors, which are important for normal gonadal function. Previous studies reported expression of inhibin in the testis of various mammals including humans [7], primates [20], rats [26], mice [23], hamsters [9], and pigs [8]. Inhibin acts on pituitary gonadotropins to suppress follicle-stimulating hormone (FSH) secretion [5] and to reduce spermatogonial numbers [25].

The pattern of inhibin expression is associated with the two distinct phases of rat Sertoli cells [10]. The first phase is related to an increase in circulating FSH levels [10], which induce Sertoli cell proliferation. The second phase is related to the increasing levels of FSH that are present during pubertal maturation [2,10,24]. Inhibin provides a negative feedback signal that downregulates the secretion of FSH [5,17]. In addition, inhibin α isotype knockout mice show testicular stromal tumors and arrest of gametogenesis [12,18]. On the other hand, transgenic mice overexpressing the inhibin A subunit or the inhibin α isotype have small testes and a reduced level of spermatogenesis [13]. This suggests that inhibin isotypes may regulate testicular maturation along with FSH. The secretion of inhibin is restricted primarily to Sertoli cells in rat testis [16]. Spermatogenic cells in the seminiferous tubules are capable of modulating the expression of inhibin in Sertoli cells both

in vitro [4,19] and *in vivo* [1,6]. Therefore, differential expression of inhibin isotypes might be observed in seminiferous tubules in mice during testicular development.

This study examined the level and cellular localization of inhibin isotypes, α, β_A and β_B, in the testis of mice during postnatal development in order to determine if inhibin is associated with testicular maturation.

Materials and Methods

Animals and tissue preparation

ICR mice used in this experiment were obtained from the animal center at the Korea Research Institute of Bioscience and Biotechnology. Mice were housed in a room maintained under the following conditions: a temperature of 23 ± 2°C, a relative humidity of 50 ± 5%, with artificial lighting from 08:00 to 20:00 and 13-18 air changes per h. The mice were fed a standard animal diet. Three mice at postnatal days (PNDs) 1, 3, 6, 18, 48 and 120 were obtained from the same litters.

Mice were sacrificed and testes were immediately removed (n = 3). A sample of the testes was embedded in paraffin wax after routine fixation in 10% buffered formalin. Paraffin sections (5 μm thick) were used in all immunostaining experiments. The opposite testis was snap-frozen and stored for immunoblot analysis. All experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (USA).

Antisera

Rabbit polyclonal anti-inhibin α (H-134), β_A (H-120) and β_B (H-110) antibodies were obtained from Santa Cruz Biotechnology (USA). Mouse monoclonal anti-beta-actin and vimentin antibodies were purchased from Sigma (USA) and Neomarkers (USA), respectively.

Western blot analysis

Testes tissues were immersed quickly in buffer H (50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM PMSF, pH 7.4), and sonicated for 10 sec. The homogenate was transferred to microtubes and centrifuged at 19,340 × g for 10 min. The supernatant was then harvested. For the immunoblot assay, the supernatant was loaded into individual lanes of 10% sodium dodecyl (lauryl) sulfate-polyacrylamide gels, electrophoresed and immunoblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, USA). The residual binding sites on the membrane were blocked by incubation with 5% nonfat milk in phosphate-buffered saline (PBS, pH 7.4) for 1 h. Subsequently, the membrane was incubated overnight at 4°C with rabbit polyclonal anti-inhibin-α, β_A and β_B antibodies (1 : 1,000 dilution). After extensive washing and incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1 : 20,000 dilution; Pierce, USA), signals were visualized using chemiluminescence (Super Signal West Pico; Pierce, USA). For normalization purposes, membranes were re-probed with antibodies against beta-actin (1 : 20,000 dilution; Sigma, USA). Several exposure times were used to obtain signals in the linear range. The bands were quantified using Scion Image Beta 4.0.2 for Windows XP software (Scion, USA). The data were analyzed using one-way ANOVA followed by a Student-Newman-Keuls post hoc test for multiple comparisons. In all cases, a p value < 0.05 was considered significant.

Immunofluorescence

Paraffin-embedded sections of testes (5 μm) were deparaffinized, treated with a citrate buffer (0.01 M, pH 6.0) in a microwave for 20 min, and then treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block

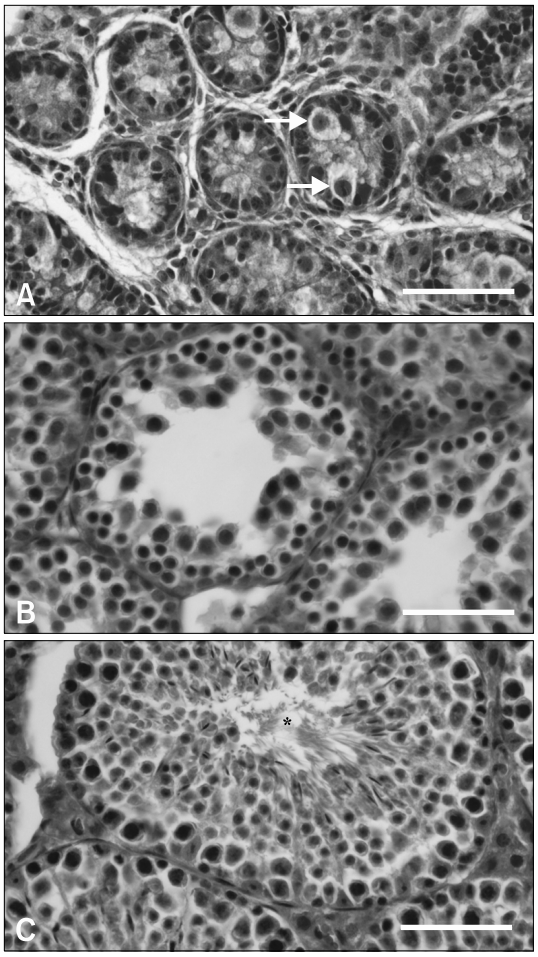

Fig. 1. Light micrographs of the mouse testes at postnatal day (PND) 1 (A), PND 18 (B), and PND 48 (C). The arrows in A indicate gonocytes in undifferentiated seminiferous epithelium. The asterisk in C indicates the defined lumens of the tubules including mature sperm cells. H&E stain. Scale bars = 40 μm.

endogenous peroxidase activity. After three washes with PBS, sections were incubated with 10% normal goat serum, and then incubated with rabbit monoclonal inhibin α, β_A and β_B (1 : 100 dilution) for 1 h at room temperature. The immunoreactivity was visualized using fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1 : 50 dilution; Sigma, USA). Cell phenotypes of inhibin α, β_A and β_B expression were examined by double label immunofluorescence using cell-type-specific markers, including vimentin (1 : 500 dilution) for the Sertoli and interstitial cells. First, the paraffin sections were reacted with primary rabbit anti-inhibin α, β_A and β_B followed by FITC-labeled goat anti-rabbit IgG (1 : 50 dilution; Sigma, USA). Slides were then incubated with mouse vimentin followed by tetramethyl rhodamine isothiocyanate-labeled goat anti-mouse IgG (1 : 50 dilution; Sigma, USA).

Results

Histological finding of the mouse testis during postnatal development

The testis at PND 48-120 showed an increase in the height of the seminiferous epithelium and the defined lumens of the tubules including mature sperm cells (Fig. 1C), while the tubules at PND 1-18 were largely undifferentiated (Figs. 1A and B). As shown in Fig. 1C, there was an abundant population of interstitial cells in the testis at PND 48. The seminiferous tubules contained primary spermatocytes, spermatids and Sertoli cells at various stages. This suggests that sexual maturation in this experimental animal occurs

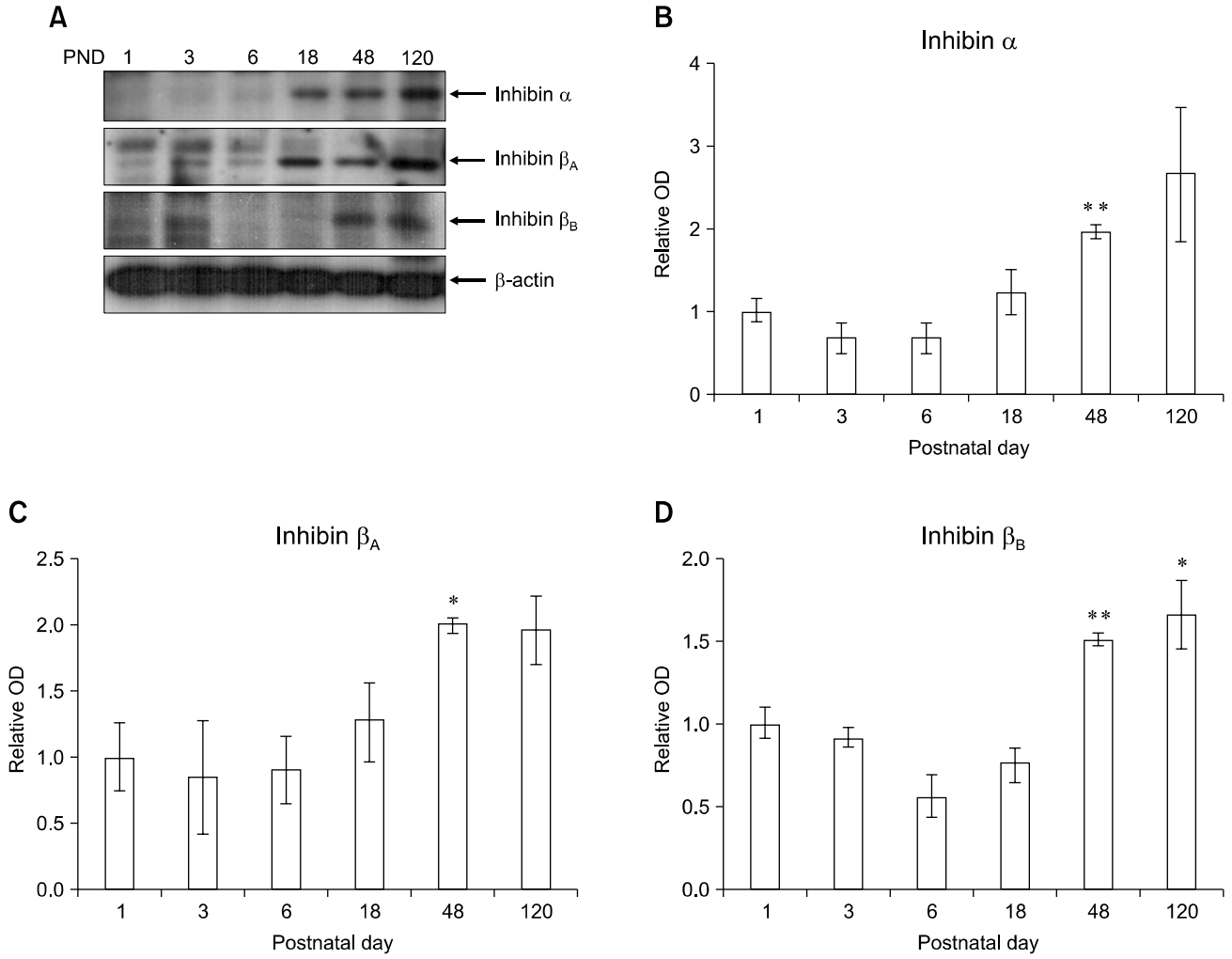

Fig. 2. Expression of inhibin isotypes α, β_A and β_B in mouse testis increased progressively with postnatal age. Photographs: Representative photographs of Western blots for inhibin isotypes α, β_A and β_B and beta-actin (A). Arrowheads indicate the positions of the inhibin isotypes (40 ~ 47 kDa) and beta-actin (45 kDa). Minor bands at various molecular weights were detected on the immunoblots for the inhibin isotypes α, β_A and β_B. Bar graph: The results of densitometric data analysis (mean ± SE, n = 3 mice/group). The relative expression levels of the inhibin isotypes were calculated after normalization to the beta-actin band from three different samples. The value for the testis at postnatal day (PND) 1 was arbitrarily defined as 1 (B, C and D, graphs). *$p < 0.05$, **$p < 0.01$ vs. PND 1-6.

between PND 18 and 48.

Temporal expression pattern of the inhibin isotypes α, β_A and β_B during the postnatal development of mouse testis

The protein levels of the inhibin α, β_A and β_B isotypes in the testes during postnatal development were analyzed semiquantitatively by Western blotting to determine the developmental changes in the inhibin isotypes.

As shown in Fig. 2, a low intensity signal for inhibin α expression was detected in the testis at days 1-6 after birth. The level gradually increased at PND 18 to 120, and there was a significant peak (approximately 2 fold, $p < 0.01$ vs. PND 1-6) at PND 48 (Figs. 2A and B). A low level of inhibin β_A expression was observed in the early phase of development (PND 1-6). The level increased and showed a significant peak (approximately 2 fold, $p < 0.05$ vs. PND 1 and 6) at day 48 after birth (Figs. 2A and C). A low intensity signal for inhibin β_B expression was detected in the testis at PND 1-6. The level increased at PND 48-120, and there were substantial levels at both PND 48 (approximately 1.5 fold, $p < 0.01$ vs. PND 1-18) and PND 120 (approximately 1.6 fold, $p < 0.05$ vs. PND 1-18) (Figs. 2A and D).

Immunofluorescent detection of inhibin α, β_A and β_B in mice testis

At PND 1-6, there was little immunoreactivity for inhibin α, β_A and β_B subunits in testicular cells (data not shown). Inhibin α expression (Fig. 3A) was observed in cytoplasmic processes of vimentin-positive Sertoli cells surrounding spermatogenic cells (Fig. 3B) at PND 18-120. Immunoreactivity for inhibin β_A was observed in the interstitial and spermatogenic cells (Fig. 3C) during PND 48-120. Inhibin β_B immunoreactivity was observed mainly in cell membranes of some spermatogonia in the seminiferous tubules as well as in the interstitial cells after PND 48 (Fig. 3D).

Discussion

This study shows a gradual increase in the expression of inhibin isotypes, α, β_A and β_B, in the testis of mice during postnatal development. Each inhibin isotype was localized differentially in testicular cells of the testes between PNDs 18-120. However, expression of these isotypes were rarely observed in testes during the early phase of postnatal development (PND 1-6).

In this study, histological examination of the development of mouse testis showed that sexual maturation is acquired between PND 18 and 48. This suggests that the two major functions of the sexually matured testis, spermatogenesis and generation of sexual hormones, were accomplished between PND 18 and 48. During this phase, protein levels of the three isotypes of inhibin in the testis also increased.

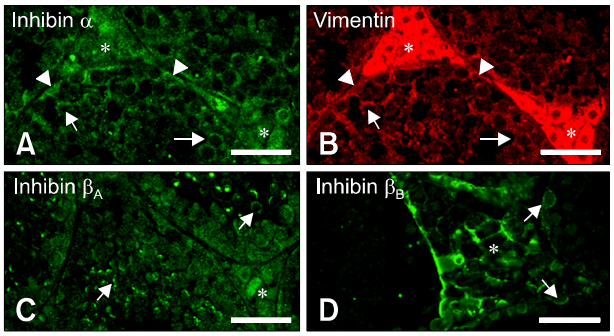

Fig. 3. Immunofluorescent localization of inhibin α, β_A, and β_B isotypes in mouse testis at postnatal days 48. (A and B) Double-immunofluorescent staining in the same section showed the co-localization of inhibin α with vimentin in cell bodies of Sertoli cells (arrowheads), the cytoplasmic process of Sertoli cells (arrows) and in interstitial spaces (asterisks). (C) Immunofluorescent localization of the inhibin β_A subunit was observed in the cell membrane of some spermatogenic cells (arrows) as well as in the interstitial cells (asterisk). (D) Immunofluorescent localization of the inhibin β_B subunit was observed mainly in cell membranes of interstitial cells (asterisk) as well as in some spermatogonia (arrows). Scale bars = 30 μm.

The histological findings in the sexual maturation of developing mouse testis are consistent with those of a previous report [23].

In this study, protein levels of the inhibin isotypes (α, β_A and β_B), were analyzed by western blotting. Low intensities of the isotypes were detected in the early phase, but the levels increased gradually during sexual maturation (PND 18 to 48). Immunohistochemical results showed that expression of inhibin isotypes increased gradually during postnatal development of mouse testis, mainly in the Sertoli and interstitial cells. Previously, it had not been reported that mRNAs for the α, β_A and β_B isotypes were closely associated with testicular maturation [14,22,23]. The level of FSH increased in rats during pubertal maturation [2,10,24]. Inhibin provides a negative feedback signal that regulates FSH secretion [5,17]. Therefore, the maturation of Sertoli cells by FSH stimulation promotes the expression of inhibin isotypes. Hence, inhibin regulates the development of Sertoli cells and spermatogenesis in mouse testis.

In this study, inhibin α immunoreactivity was detected mainly in Sertoli cells from puberty to adulthood, as previously indicated for rat testis [16]. In addition, expression of inhibin β_A and β_B subunits was detected in interstitial and spermatogenic cells in the testes of mice from puberty to adulthood. Several studies have reported that the differential expression in various types of testicular cells depends on the animal species [3,8,9,15,17]. Therefore, further studies will be needed to determine the functional role of inhibin via local or paracrine secretion among testicular cells.

In conclusion, expression of the inhibin isotypes α, β_A and β_B, in the testes of mice gradually increased during postnatal development. Each isotype was localized differentially in

testicular cells during maturation. The expression of inhibin isotypes in the testis of mice increased progressively with postnatal age, which suggests that inhibin is associated with a negative feedback signal for FSH during testicular maturation.

Acknowledgments

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