Changes in Circulating and Testicular Levels of Inhibin A and B During Postnatal Development in Bulls

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Abstract. We investigated testicular and circulating levels of dimeric inhibins in Holstein bulls from the infantile to postpubertal periods (5 to 50 weeks of age) and examined the relationship between the profiles of circulating dimeric inhibins and FSH. Concentrations of total inhibin and inhibin B in the testis were highest at 4 to 5 weeks of age but decreased gradually as the bulls aged. Testicular inhibin A levels showed a gradual decline to a nadir at 15 to 26 weeks of age, but by 39 weeks, they were high again. The contents of total inhibin, inhibin A, and inhibin B per testis generally increased with age. Fractionation of testicular homogenates obtained from 15-week-old bulls by a combination of immunoaffinity chromatography and SDS-PAGE confirmed the presence of two major molecular weight forms (32 and 45 kDa) of dimeric inhibins in the testes. Circulating levels of total inhibin and inhibin A showed a significant increase in bulls at around 10 to 14 weeks of age compared to the levels between 5 and 7 weeks of age but decreased thereafter. However, immunoreactivity for inhibin B was not detected in the peripheral circulation, probably because of low sensitivity of the inhibin B assays. The concentrations of plasma FSH were high at 5 weeks of age but declined to lower levels between 11 and 40 weeks, and then increased from 41 weeks onward. There was no significant correlation between the plasma levels of FSH and inhibin A or total inhibin. The results clearly indicate that the bull testis produces inhibin A and B and secretes at least inhibin A into the circulation during postnatal development. However, the profile of circulating FSH in bulls shows no reciprocal relationship with the inhibin A or total inhibin profile during the postnatal period.

Key words: Bulls, Circulation, Inhibin A, Inhibin B, Postnatal period, Testis

Follicle-stimulating hormone is a regulator of testicular growth and function, and its secretion is regulated by a balance between the stimulatory effects of gonadotropin-releasing hormone from the hypothalamus and the inhibitory effects of hormones secreted by the testis. Inhibin is a dimeric protein composed of an α subunit and either a βA subunit (inhibin A) or βB subunit (inhibin B); it is produced in the gonads and suppresses FSH secretion in cultured pituitary cells. In vivo immunoneutralization studies of inhibin have revealed that inhibin functions as a negative regulator of FSH secretion in male rats [1], hamsters [2], goats [3], and cattle [4–7].

Previous studies that examined the relationship

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between circulating FSH and inhibin in developing bulls reached contradictory conclusions; two studies found a negative correlation between the plasma levels of inhibin and FSH from 10 to 36 weeks of age [8] or from 2.5 to 108 months [9], whereas Miyamoto et al. [10] found a positive correlation between 1 and 8 months. However, the radioimmunoassays (RIAs) used in the above studies were based on antisera directed against the α subunits of inhibin. They therefore did not discriminate the dimeric inhibins, which have FSH-suppressing activity, from the free α subunits, which have no FSH-suppressing activity and exist in the ovary [11–13], testis [14, 15], and peripheral circulation [11].

To overcome this major limitation in inhibin RIAs, the sandwich-type enzyme-linked immunosorbent assay (ELISA; [16]) or time-resolved immunofluorometric assay (Tr-IFMA; [17]) for dimeric inhibins has been developed. In many adult male animals studied to date, the predominant inhibin isoform in the circulation is inhibin B (humans: [18]; non-human primates: [19, 20]; rats: [21, 22]; hamsters: [23]; miniature pigs: [24]). A few exceptions to this rule are adult rams and pigs, the testes of which secrete inhibin A into the circulation [25, 26]. Our previous study [15] demonstrated that the bull testis produces inhibin A and B from the infantile to postpubertal periods; however, the profiles of circulating dimeric inhibins and their relationship with the FSH profile have not been elucidated in bulls.

Therefore, the aims of this study were to clarify the changes in the testicular and circulating levels of dimeric inhibins using specific Tr-IFMAs and to examine the relationship between FSH and dimeric inhibin during the postnatal period.

**Materials and Methods**

**Animals and sampling**

The protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Japan. Five Holstein bulls were weighed bi-weekly and subjected to weekly blood sampling from 5 to 50 weeks of age. The body weights of the 5 Holstein bulls increased from 56.3 ± 3.5 kg (mean ± SEM, n=5) at 5 weeks of age to 484.3 ± 38.5 kg at 50 weeks. Plasma was recovered after centrifugation of blood and stored at ~30 C. Testes were surgically obtained from between 5 and 10 Holstein bulls at 4 to 5, 7 to 9, 15, 17 to 19, 21 to 26, 31 to 32, 39 to 42, and 49 to 55 weeks of age. The testes were homogenized in a 10-fold concentration of Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5 mM EDTA and 0.1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma, St. Louis, MO, USA) and stored at ~80 C.

**Time-resolved fluoroimmunoassay for total inhibin**

The concentrations of total inhibin were determined by a competitive immunoassay using europium (Eu)-labeled bovine inhibin A as a probe [27]. In the fluoroimmunoassay (FIA) of total inhibin, anti-bovine inhibin serum (TNDH-1: [28]) was used as a primary antibody. Bovine 32-kDa inhibin A purified from follicular fluid was used for Eu-labeling and as a reference standard (anti-inhibin serum was provided by Dr. K. Taya, Tokyo University of Agriculture and Technology, Fuchu, Japan, and bovine 32-kDa inhibin A was provided by Dr. Y. Hasegawa, Kitasato University, Towada, Japan). This assay recognizes several molecular weight forms of dimeric inhibin A as well as 26-kDa pro-αC in bovine follicular fluid (bFF) [27, 29]. The assay also recognizes porcine 29-kDa inhibin B in a dose-dependent manner. Thus, the FIA measures both inhibin A and inhibin B in addition to the free α subunits. The detection limit of the FIA was 0.078 ng/ml. The intra- and interassay coefficients of variation (CVs) were 9.6 % and 10.7 %, respectively.

**Time-resolved immunofluorometric assay for inhibin A**

The concentrations of inhibin A were determined by an immunofluorometric assay (IFMA) using purified polyclonal antibodies to the α and βA subunits of bovine inhibin A, as described previously [17]. Bovine 32-kDa inhibin A was used as a reference standard (bovine 32-kDa inhibin A was provided by Dr. Y. Hasegawa, Kitasato University, Towada, Aomori, Japan). Inhibin A IFMA showed very low cross-reactivity with human recombinant inhibin B (<0.01%) and with activin A (<0.01%), activin B (<0.01%), activin AB (<0.01%), and pro-αC (<1%) purified from bovine follicular fluid. This assay recognizes several molecular weight forms of dimeric inhibin A in bFF [17, 29]. The sensitivity limit of the inhibin A IFMA
was 3.3 pg/ml. The intra- and interassay CVs were 11.0% and 14.9%, respectively.

**Tr-IFMA for inhibin B**

The concentrations of inhibin B were determined by IFMA using purified polyclonal antibodies to the α and βB subunits of bovine inhibin, as described previously [15]. Porcine 29-kDa inhibin B purified from follicular fluid was used as a reference standard (porcine 29-kDa inhibin B was provided by Dr. Y. Hasegawa). Inhibin B IFMA showed very low cross-reactivity with inhibin A (<1.5 %), activin A (<0.5%), activin B (<0.5%), activin AB (<2.0%), and pro-αC (<0.5%) purified from bFF. This assay recognizes several molecular weight forms of dimeric inhibin B in the bovine testis [15, 29]. The detection limit of the assay was 0.39 ng/ml. The intra- and interassay CVs were 9.8% and 15.7%, respectively.

**Tr-FIA for bovine FSH**

The concentrations of FSH in the plasma were determined by competitive immunoassays using Eu-labeled FSH as a probe [17]. In the FIA of bovine FSH, anti-bovine FSH β-subunit serum (USDA-5-pool, [30]) was used as a primary antibody, USDA-bFSH-I2 was used for Eu-labeling and USDA-bFSH-I2 was used as a reference standard [Assay materials were provided by the United States Department of Agriculture (USDA) Animal Hormone Program, Germplasm and Gamete Physiology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD, USA]. The intra- and interassay CVs were 10.1% and 17.2%, respectively.

**Isolation of inhibin from testicular homogenates**

Pooled testicular homogenates obtained at 15 weeks of age were fractionated by two-cycle immunoaffinity chromatography as described previously [15], to confirm the results obtained from the measurements of testicular inhibin. An aliquot of the immunoaffinity extract containing 10 µg total inhibin, as determined by fluoroimmunoassay, was subjected to 12.5% SDS-PAGE. The gel was cut into 1.0-mm slices, and inhibin was extracted from each gel slice in 1 ml of TBS containing EDTA and CHAPS. The gel eluates were stored at ~80°C for subsequent inhibin immunoassays.

**Immunoblotting**

The above immunoaffinity extract of 15-week-old testes were subjected to immunoblotting using inhibin α antibody as described previously [5]. The amount of total inhibin subjected to SDS-PAGE, as determined by FIA, was approximately 150 ng/ lane.

**Statistical analyses**

Hormone concentrations in the plasma were subjected to ANOVA for repeated measures [31]. Changes in the testicular concentrations of hormones were subjected to one-way ANOVA. When a significant effect was obtained by ANOVA, the significance of the difference between means was determined by Duncan’s multiple range test. Regression analysis between the plasma concentrations of inhibin and FSH was also performed. All data were analyzed using the General Linear Models or REG Procedure of Statistical Analysis Systems [32]. A value of P<0.05 was considered to denote statistical significance.

**Results**

**Dose-response curves of plasma and testicular samples in immunoassays for inhibin**

Serial dilutions of testicular homogenates and immunoaffinity extracts from the testis showed dose-response curves that were parallel to the standard curves generated with inhibin A in the FIA (Fig. 1), and with inhibin A or inhibin B in the respective IFMAs (Figs. 2 and 3). The dose-dependent curves of serially diluted peripheral plasma were parallel to the standard curves produced with inhibin A in the FIA (Fig. 1), and with inhibin A in the IFMA (Fig. 2); however, no specific immune reaction was observed for plasma in the inhibin B IFMA (data not shown).

**Testicular concentrations of inhibin**

The concentrations of total inhibin, in terms of amount per gram of testis, were highest at 4 to 5 weeks of age (14.0 ± 0.7 µg/g testis, mean ± SEM, n=5), but steadily declined (P<0.01) to a minimum at 49 to 55 weeks of age (1.6 ± 0.06 µg/g, n=9) (Fig. 4a). Inhibin A concentrations were 135.8 ± 5.4 ng/g at 4 to 5 weeks of age and then decreased (P<0.05) to a nadir of approximately 70 ng/g between 15 and 21 to 26 weeks of age (Fig. 4b). Inhibin A then
rose again from 31 to 32 weeks of age and returned to high levels between 39 to 42 and 49 to 55 weeks of age. Testicular inhibin B concentrations were highest at 4 to 5 weeks of age (85.1 ± 4.7 ng/g testis) but decreased (P<0.01) with age (Fig. 4c). Total inhibin contents per testis did not change significantly until 31 to 32 weeks of age but increased (P<0.05) to greater than 200 µg/testis between 39 to 42 and 49 to 55 weeks of age (Fig. 4a). Age-related changes in the testicular contents of inhibin A and inhibin B were similar to those in total inhibin contents (Figs. 4b and c).

Isolation of inhibin from testicular homogenates

In the Holstein bull testis at 15 weeks of age, high immunoreactivity for total inhibin was observed at molecular weights of approximately 25 and 45 kDa (Fig. 5a). IFMA for inhibin A recognized 45- and 31-kDa peaks (Fig. 5b), whereas inhibin B IFMA detected only a 45-kDa peak (Fig. 5c). Immunoblotting using the α subunit antibody detected the presence of clear bands of 23, 25, 32, 39, 43, and 45 kDa in the same extract obtained from 15-week-old testes with the two-cycle immunoaffinity chromatography (Fig. 5).
Plasma concentrations of inhibin and FSH

Plasma concentrations of total inhibin ranged from 5.5 to 6.5 ng/ml between 5 and 8 weeks of age but increased (P<0.05) to 8.6 ± 0.7 ng/ml (mean ± SEM, n=5) by 11 weeks of age and remained high until 13 weeks of age (Fig. 6a). Total inhibin levels gradually decreased (P<0.05) with age between 20 and 50 weeks of age. The concentration of inhibin A was approximately 1.2 ng/ml between 5 and 8 weeks of age and peaked (P<0.05) at greater than 1.8 ng/ml between 10 and 14 weeks of age, but decreased with age thereafter (Fig. 6b). Inhibin B levels in the circulation were below the detection limit of the assay at all ages (data not shown). The plasma concentration of FSH was high (162.1 ± 25.1 pg/ml) at 5 weeks of age but had decreased to 84.6 ± 23.0 pg/ml by 10 weeks of age (Fig. 6c). FSH levels remained low between 11 and 40 weeks of age but increased between 41 and 50 weeks of age, except at 44 and 49 weeks of age. There were no significant correlations between the circulating levels of FSH and inhibin A (r=0.086, P>0.1) or total inhibin (r=0.05, P>0.1) from 5 to 50 weeks of age.

Discussion

Our results clearly indicate that 1) inhibin A was present in the testis and circulation of bulls from the infantile to postpubertal periods, whereas inhibin B was detected in the testis but not in the circulation; and 2) no relationship existed between the circulating levels of FSH and inhibin A or total inhibin (r=0.05, P>0.1) from 5 to 50 weeks of age.
consistent with our previous findings [15], but our present results clearly indicate that inhibin A is secreted into the peripheral circulation of bulls from the infantile to postpubertal periods. On the other hand, inhibin B was not detectable in the peripheral circulation despite its existence in the testis. The most probable explanation for this is the lower sensitivity of inhibin B IFMA compared with that of inhibin A IFMA. Previous findings have demonstrated that the predominant inhibin isoform in the circulation is inhibin B in adult male humans [18], non-human primates [19, 20], rats [21, 22], hamsters [23], and miniature pigs [24]. Although we cannot draw a conclusion about inhibin B levels in the circulation, the bull is another example in which inhibin A is detected in the circulation, as it is in rams [25] and boars [26].

The concentrations of total inhibin in the testis were much greater than those of inhibin A or inhibin B throughout the experiment. This indicates that the bovine testis produces large amounts of the free α subunits, which is consistent with the presence of 20–25 kDa materials cross-reacted with the inhibin α antibody. Testicular concentrations of total inhibin were high at 4 to 5 weeks of age but then decreased with age, a result similar to previous findings in developing bulls [33] and boars [26]. This age-related change in testicular total inhibin levels parallels the decrease in immunoreactivity for inhibin α subunits in bovine Sertoli cells [5, 15, 33] and indicates that total production of inhibin-related proteins in Sertoli cells decreases with age. The changes in testicular inhibin B levels were similar to those in the total inhibin levels, but the inhibin A levels showed a different pattern; high inhibin A levels were observed between 39 and 55 weeks of age following a decline to a nadir between 15 and 26 weeks of age. Miyamoto et al. [10] demonstrated that inhibin bioactivity in the testes of Japanese beef cattle, as determined by suppression of FSH secretion from cultured rat pituitary cells, was high during the first 3 months of life but low between 4 and 6 months of age, then increased again between 7 and 8 months of age. Our profiles of the inhibin A levels, or our profiles of inhibin A plus B levels combined, agree well with the changes in inhibin bioactivity in a previous study [10]. At present, the mechanisms involved in the increase in inhibin A production in the testis toward puberty are not clear, but this increase may reflect functional changes in Sertoli cells in the form of transition from undifferentiated types to matured ones; these changes occur between 24 and 28 weeks of age [34, 35].

The circulating levels of total inhibin and inhibin A reached a plateau by 14 weeks of age and then decreased as the bulls aged, and the changes in total inhibin were similar to previous findings [9, 10]. We can expect that a combination of testicular inhibin content and the testis weight to body weight ratio contributes to changes in the circulating concentrations of inhibin. In our bulls, the amounts of total inhibin and inhibin A per whole testis generally increased with age, and it has been shown previously that the ratio of testis weight to body weight increases toward the pubertal period [34]. However, a major discrepancy between the circulating inhibin levels and testicular inhibin levels appeared as the bulls aged. In bull testes, the lumen of the seminiferous

![Fig. 6. Changes in plasma concentrations of (a) total inhibin, (b) inhibin A, and (c) FSH in bulls from 5 to 50 weeks after birth. Values are means ± SEM (n=5 bulls).](image)
tubules, evidence of fluid secretion and formation of the blood-testis barrier, appears between 24 and 28 weeks of age [34, 35]. In adult rams, Voglmayr et al. [36] detected a much higher level of immunoreactive inhibin in the rete testis than in the testicular lymph or peripheral blood. These findings suggest that, after formation of the blood-testis barrier, inhibin is released into the seminiferous tubules and some of it reaches the general circulation by resorption from the rete testis fluid via the lymphatic vessels. This is probably the cause of the decrease in circulating inhibin levels in bulls after 20 weeks of age, in spite of the increase in the testicular contents of inhibin.

We found no correlation between the FSH profile and the inhibin A or total inhibin profile; this finding is not consistent with previous reports of a negative [8, 9] or positive correlation [10]. The discrepancy between these previous studies and ours is not readily explained, but our determination of the inhibin and FSH profiles was more precise because we used more frequent blood sampling (1-week intervals) than was used in these other studies (2- to 12-week intervals). On the other hand, immunoneutralization of inhibin increases FSH secretion during the infantile [5], prepubertal [6], and postpubertal [4, 7] periods, thus providing direct evidence that inhibin functions as a negative regulator of FSH secretion in bulls. Our analysis and a previous study [15] of inhibin molecules in the testis clearly indicate that the bovine testis produces several molecular weight forms of dimeric inhibin A and B. Our results strongly suggest that inhibin A, and probably inhibin B, secreted from the Sertoli cells continuously tunes FSH secretion, although a clear relationship was not found between the circulating levels of inhibin A and FSH; this differs from the case in cows, where there is a clear inverse relationship between inhibin and FSH levels associated with dynamic changes in the growth and regression of follicles [17, 37, 38].

In summary, Tr-IFMAs revealed that inhibin A was present in the circulation of bulls during the postneonatal period. However, no relationship was found between the circulating inhibin A and FSH profiles, despite the production of biologically active forms of inhibin in the testis.

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