Immunohistochemical Localization of Inhibin/Activin Subunits in the Wild Ground Squirrel (Citellus dauricus brandt) Ovary

Xia SHENG1), Jiaju WENG2), Haolin ZHANG1), Xiaonan LI1), Mengyuan ZHANG1), Meiyu XU1), Qiang WENG1),3), Gen WATANABE3),4) and Kazuyoshi TAYA3),4)

1) College of Biological Science and Technology, Beijing Forestry University, Beijing 100083, PR China
2) School of Basic Medical Science, Peking University, Beijing 100083, PR China
3) Laboratory of Veterinary Physiology, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
4) Department of Basic Science, United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

Abstract. The intraovarian function of gonadally produced inhibin and activin has been extensively studied in experimental models for decades, yet their presence and function have been rarely reported in wild rodents. With our seasonal breeding model, the wild ground squirrel, we aimed to investigate the possible roles of these peptides in the seasonal folliculogenesis. Immunohistochemical staining and Western blotting have been used to detect the cellular localization and expression patterns of inhibin/activin subunits (α, βA and βB). In the breeding season ovary, all three subunits were present in granulosa cells, theca cells of antral follicles and interstitial cells, with the strongest immunostaining in granulosa cells. Following ovulation, the corpora lutea become a major site of inhibin/activin synthesis. In the nonbreeding season ovary, inhibin/activin α and βA subunits were weakly immunopositive in granulosa cells of early stage follicles, while βB subunit was undetectable. The expression level of inhibin/activin subunit proteins were generally higher in the ovaries of the breeding season, and then decreased to a relatively low level during the nonbreeding season. The dynamic expression of inhibin/activin subunits indicated that they might play important paracrine and/or autocrine roles during the seasonal folliculogenesis of the wild ground squirrel.

Key words: Folliculogenesis, Immunohistochemistry, Inhibin/activin subunits, Ovary, Wild ground squirrel

Inhibin and activin are prototypical members of the transforming growth factor β (TGFβ) superfamily of ligands and receptors, which are temporally and spatially widespread and functionally diverse [1–3]. Inhibin is composed of an α-subunit disulfide linked to a β subunit, and the particular isoform of inhibin is named for the β subunit present in that molecule (inhibin A, α/βA, and inhibit B, α/βB). Activins are homodimers of the inhibin β subunits (activin A, α/βA; activin B, α/βB, and activin AB, βA/βB) [4–6]. Biological roles for activin have been proposed in a number of reproductive organs including the gonads, pituitary, and uterus, where it regulates processes such as folliculogenesis, spermatogenesis, and pregnancy, while the primary role of inhibin appears to be antagonism of inhibin/activin signaling in many cells, including pituitary gonadotrope FSH synthesis and ovarian theca and testicular Leydig cell androgen production [7–9]. In the ovary, inhibin and activin, mainly produced by granulosa cells, are thought to be involved in many intraovarian roles during folliculogenesis [10]. Activin and several other TGF-β superfamily ligands play key roles in germ cell survival, in primordial follicle assembly and in follicle growth from the preantral to mid-antral stages, and exert paracrine actions on theca cells to attenuate LH-dependent androgen production in small- to medium-sized antral follicles [11,12]. Changes in intrafollicular activins may contribute to dominant follicle selection by modulating both FSH- and IGF-dependent signaling pathways in granulosa cells. Activin may also play a positive role in oocyte maturation and acquisition of developmental competence. In addition to its endocrine feedback to suppress FSH secretion, increased output of inhibin by the selected dominant follicle may upregulate LH-induced androgen secretion that is required to sustain a high level of estradiol secretion during the preovulatory phase [11–13].

On the other hand, accumulating information has also been gained on the role of the inhibin/activin system in ovarian regulation in nonmammalian vertebrates, which exhibits a considerable overlap with that in mammals [12,14]. However, little is known in terms of the functional involvement of the inhibin/activin system in seasonal folliculogenesis in a wild rodent. The wild ground squirrel (Citellus dauricus Brandt) is a typical seasonal breeder with a short sexually active period in April and May, followed by a long period of sexual dormancy from June to the following March [15]. Previously, we have detected the immunoreactivity of inhibin/activin subunits, activin type II receptor, activin-related SMADs and steroidogenic enzymes (P450c17 and P450arom) in the testis of the wild ground squirrel and revealed an important paracrine role of the activin signal in seasonal spermatogenesis [16–18]. The present study aimed to investigate the immunoreactivity of inhibin/activin subunits in the ovarian recrudescence and regression during the breeding and...
nonbreeding seasons, and to explore the potential roles of the most substantial ovarian peptide hormones in the seasonal folliculogenesis of the wild ground squirrel.

**Materials and Methods**

**Animals**

Fourteen wild female ground squirrels thought to be adult based on their body weights (242–412 g) were captured in April (breeding season) and July (nonbreeding season) of 2010 in Hebei Province, P.R. China. All procedures involving animals were carried out in accordance with the Policy on the Care and Use of Animals, approved by the Ethics Committee of Beijing Forestry University, and the Department of Agriculture of Hebei Province, P.R. China (JNZF11/2007). The animals were euthanized with ether before decapitation. The ovaries were excised from each body, and every obtained ovary was cut into 2 portions; one portion was fixed in 4% paraformaldehyde in 0.05 M PBS (pH 7.4) for histological and immunohistochemical observations, and the other was immediately stored at −80 °C until it was used for Western blotting detection.

**Histology**

Ovarian samples were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections (4 μm) were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA). Sections were stained with hematoxylin-eosin (HE) for observations of general histology. The sections were screened using an Olympus photomicroscope with a ×20 objective lens and imaged with the Image-Pro Plus 4.5 software (Media Cybernetics, Bethesda, MD, USA).

**Immunohistochemistry**

The sections were incubated with 10% normal goat serum to reduce background staining caused by the secondary antibody. The sections were then incubated with primary antibodies (1:1000 or 1:2000) raised against porcine inhibin α chain (1–30)-NH₂ conjugated to rabbit serum albumin, porcine inhibin/activin βA (81–113)-NH₂ (#305-24D) and cyclic acetyl human inhibin/activin βB (81–113)-NH₂ (#305-25D) [19] for 12 h at room temperature. The inhibin α subunit peptide was kindly provided by Dr N Ling (Neuroendocrine, San Diego, CA, USA); the antibodies of inhibin/activin βA (81–113)-NH₂ (#305-24D) and cyclic acetyl human inhibin/activin βB (81–113)-NH₂ (#305-25D) [19] for 12 h. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 60 min. Finally, the membrane was colored with 25 mg 3,3-diaminobenzidine (Wako) solution in 25 ml TBS-T buffer, pH 7.6. β-actin was used as the endogenous control. Negative control blots were performed for every experiment using identical samples with water instead of the primary antibody. Signals were quantified by densitometric analysis using the Quantity One software (Version 4.5, Bio-Rad Laboratories, Hercules, CA, USA) and presented as the relative density. The protein expression levels of both breeding and nonbreeding seasons were normalized to that of the β-actin.

**Western blotting**

Ovarian tissue was diced into small pieces using a clean razor blade. Tissue was homogenized in a homogenizer containing 300 μl of 10 mg/ml PMSF stock and incubated on ice for 30 min, with the temperature maintained at 4 °C throughout all procedures. Homogenates were centrifuged at 12,000× g for 10 min at 4 °C. Twenty-five-microgram protein extracts (measured by Lowry Protein Assay) were mixed with an equal volume of 2× Laemmli sample buffer. Equal amounts of each sample were loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membranes were carried out using a 1:1000 dilution in TBS buffer (0.02 M Tris, 0.137 M NaCl, pH 7.6) of primary antibody raised against porcine inhibin α chain (1–30)-NH₂ conjugated to rabbit serum albumin, porcine inhibin/activin βA (81–113)-NH₂ (#305-24D) and cyclic acetyl human inhibin/activin βB (81–113)-NH₂ (#305-25D) [19] for 2 h. Secondary incubation of the membrane was then carried out using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 60 min. Finally, the membrane was colored with 25 mg 3,3-diaminobenzidine (Wako) solution in 25 ml TBS-T buffer (0.02 M Tris, 0.137 M NaCl, and 0.1% Tween-20, pH 7.6) plus 3 μl H₂O₂. β-actin was used as the endogenous control. Negative control blots were performed for every experiment using identical samples with water instead of the primary antibody. Signals were quantified by densitometric analysis using the Quantity One software (Version 4.5, Bio-Rad Laboratories, Hercules, CA, USA) and presented as the relative density. The protein expression levels of both breeding and nonbreeding seasons were normalized to that of the β-actin.

**Statistical analysis**

Mean values (± SD) were calculated and analyzed using one-way ANOVA. Tukey’s test was used for detection of significant differences using the SPSS computer package.

**Results**

**Morphological and histological observation of the wild ground squirrel ovaries of the breeding and nonbreeding seasons**

Ovaries of the wild ground squirrel progress from a highly active stage in the breeding season to an inactive state in the nonbreeding season. Morphologically, the size of the ovaries in the breeding season (Fig. 1a, left side) is apparently much larger than that of the β-actin. HE staining has also revealed a significant seasonal variance in terms of folliculogenesis, as shown in Fig. 1b and c. In the breeding season ovary, mature antral follicles were observed as expected, along with the corpora lutea. Meanwhile, early stage follicles (from primordial to preantral) were all present (Fig. 1a). In contrast, there were mainly primordial and primary follicles in the nonbreeding season ovary, with only a few progressing to the preantral stage. No tertiary follicle or corpus luteum was detected (Fig. 1b).
Immunohistochemical localization of inhibin/activin subunits in the wild ground squirrel ovaries of the breeding and nonbreeding seasons

Fig. 2 shows the representative immunohistochemical localization of inhibin/activin subunits in the ovaries of both the breeding and nonbreeding seasons. In the breeding season ovary, all antral follicles exhibited high levels of inhibin α and inhibin/activin βA and βB subunits in granulosa cells, with relatively low immunoreactivity of inhibin α and inhibin/activin βA in theca cells and interstitial cells (Fig. 2a, b, c, d). Strong positive cytoplasmic staining of all three subunits was observed in the corpora lutea as well (Fig. 2e, f, g). Follicles from an early (primary/secondary) stage of development were immunopositive for both β subunits in granulosa cells, but no consistent staining for the inhibin α subunit was detected (data not shown). In the nonbreeding season, a weak signal of inhibin α, moderate immunostaining of inhibin/activin βA and little to no immunoreactivities of inhibin/activin βB were found in the early antral follicles (Fig. 2i, j, k). Neither theca cells nor interstitial cells were immunopositive for the three subunits. No positive signal was detected in the control sections (Fig. 2d, h, l).

Expression level of inhibin/activin subunits proteins in wild ground squirrel ovaries of the breeding and nonbreeding seasons

The results of Western blotting analysis for the three subunits in ovaries of the breeding and nonbreeding seasons are shown in Fig. 3.
Fig. 3. Strong positive signals of inhibin α and inhibin/activin βA and βB subunits were all detected in protein extracted from ovaries in April, while in July, signals of inhibin α and inhibin/activin βA subunits were found (Fig. 3a, b, c). Bands of approximately 52 kDa for free α subunit (Fig. 3a), 15 kDa for mature βA subunit (Fig. 3b), and 56 kDa for free βB subunit and 27 kDa for mature βB subunit (Fig. 3c) were identified in the ovarian lysates, which is generally in accordance with previous reports [20–22]. It is noteworthy that the inhibin α antibody was able to detect only the precursor and that it could not detect the mature subunit as it did in other species [20]. Apparently, future research on the amino acid sequencing is called for in order to determine the exact molecular sizes of these subunits in this species. Generally, all subunits showed higher expression levels in the breeding season than those of the nonbreeding season (Fig. 4a, b, c and d). No signal was detected in each control lane in which water was used instead of primary antibody. β-actin, observed at around 42 kDa (Fig. 3d), showed relatively consistent signals in both seasons. All experiments were performed at least 3 times, and one representative experiment is shown.

Discussion

The present study demonstrated that immunoreactivities of inhibin/activin subunits were greater in the ovaries of the breeding season and then decreased to a relatively low level in the nonbreeding season, which was in accordance with the protein expression levels in the Western blotting results. These findings suggested that inhibin/activin subunits might play an essential regulatory role in the seasonal folliculogenesis of the wild ground squirrel.

Extensive studies have been focused on the localization and expression of inhibin/activin subunits (both protein and mRNA) in the ovaries of various species, such as mice [23], rats [24, 25], golden hamsters [26, 27], mares [28], guinea pigs [29], goats [30], elephants [31] macaques [32] and humans [33], implicating inhibin and activin as critical intraovarian regulators of follicle development in a variety of mammalian species. In neonatal mice, all three subunits were strongly stained in granulosa cells and theca-interstitial cells of antral follicles, and inhibin B was thought to be the main functional isoform [23]. During the rat estrous cycle, inhibin α subunit is expressed in granulosa cells of follicles at all stages, including primary to tertiary follicles; on the other hand β subunits were detected exclusively in granulosa cells of healthy tertiary follicles [25]. Inhibin synthesis increases in the follicles recruited by the secondary FSH surge that in turn suppress FSH levels during the early part of the estrous cycle [24]. In the golden hamster, α subunit mRNA was localized in granulosa cells of primary, secondary, tertiary and atretic follicles and luteal cells, while granulosa cells of large secondary and tertiary follicles were the primary site of βA and βB subunit mRNAs [27]. It
INHIBIN IN THE GROUND SQUIRREL OVARY

is now generally accepted that granulosa cells are the major ovarian cites that produce both α and β subunits. The present study expands our knowledge about inhibin/activin in the ovary of a wild rodent for the first time. The overall expression pattern of inhibin/activin subunits in the wild ground squirrel is in general agreement with the previous findings: granulosa cells of primary to tertiary follicles produce various amounts of subunits; certain theca and interstitial cells are also immunopositive for these subunits; and large follicles secrete proportionally more inhibin α subunit compared with smaller follicles. The immunolocalizations of both β subunits are similar in early stage follicles of both reproductive periods, indicating that preantral follicles have a gonadotropin-independent ability to synthesize activin. Interestingly, different from previous studies in rodents but similar to studies of primates and humans, the high expression levels of all three subunits are maintained in luteal cells of the wild ground squirrel, implying a potentially critical role of inhibin/activin elevation in progesterone secretion of this wild species [32, 34].

A changing intrafollicular balance between mutually opposing inhibin and activin has been proven to contribute to granulosa cell proliferation/differentiation, theca cell androgen synthesis and luteinization [12]. Since antral follicles are critically dependent on FSH for continued growth and survival, the follicles that eventually achieve dominance acquire augmented responsiveness to FSH. In the breeding season ovary, all inhibin/activin subunits exhibited their strongest immunoreactivities in granulosa cells of antral follicles, particularly those of the dominant follicles, reinforcing the pivotal role of the inhibin/activin system in antral follicle development, the follicle selection mechanism and somatic cell function [35]. On the other hand, it is well-known that activins and inhibins have reciprocal paracrine actions on theca cell androgen production [36, 37]. Recently, Young and McNeilly reported that activin significantly attenuates expression levels of StAR and 3βHSD, whereas inhibin acts almost exclusively by blocking the inhibitory action of activin and consequently increases androgen production by normal ovarian thecal cells [38]. Our recent results have also revealed that preantral follicles express high levels of P450c17 in theca cells and P450arom in granulosa cells, indicating high levels of androgen and estrogen synthesis (Weng et al., unpublished data). Therefore, the increased output of inhibin by the dominant follicle(s) of the wild ground squirrel may take part in the androgen secretion upregulation to guarantee enough estradiol production during the breeding season. Regarding luteinization and progesterone production, a positive role for inhibin A and/or its free α subunit and a negative role for activin A have been proposed [39, 40]. In the case of the wild ground squirrel, corpora luteal cells become a significant source of all three subunits. Similar to the findings revealed in primates and humans, they probably dimerize into inhibins rather than activins to further stimulate this process.

During the progression from primary to early-antral stage follicles, most studies to date have been confined to activin A among the several different activin isofoms [11]. The wild ground squirrel appears to be no exception: in the nonbreeding season, an immunoreactive signal for β, in granulosa cells of primary and preantral stage follicles was observed, and the result of Western blotting further confirmed this, implicating a latent local role of activin A in granulosa cell proliferating and early follicle progression of the wild ground squirrel. As mentioned above, the balance between activin and inhibin production shifts throughout follicle development, with primary to preantral follicles mainly expressing β subunits to form activins and larger more developed follicles expressing more α subunit to form inhibins [12, 41]. The findings in the wild ground squirrel were in accordance with this: weak positive signals of α subunit were preferentially detected in the larger preantral follicles in the nonbreeding season ovary, showing that a decreasing inhibin:activin ratio might also exist during the early stage of follicle development in this wild species. Even though our attempts on evaluating the serum concentration of gonadotropins (especially FSH) have not brought much information, it is highly likely that the level of FSH is significantly higher in the breeding season than that in the nonbreeding season. Future work will continue detecting the serum concentrations of gonadotropins during the annual cycle, and if confirmed as we hypothesized, the wild ground squirrel would make a useful natural model to study the difference between folliculogenesis with and without FSH support.

In conclusion, the present evidence in the wild ground squirrel showing that inhibin/activin subunits were present abundantly in granulosa cells of antral follicles and corpora lutea following ovulation, while preantral follicles of the nonbreeding season also showed weak immunoreactive α and β, subunits, strongly implicates that inhibin and activin play an essential role in the regulation of seasonal folliculogenesis in the wild ground squirrel.

Acknowledgments

The authors are grateful to Dr N Ling (Neuroendocrine Inc., San Diego, CA, USA) for providing [Tyr30] inhibin-α-(1–30) and Dr W Vale (Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA, USA) for providing antisera against inhibin β and β subunits. We thank W Xu (Tsinghua University, China) for her help with Western blot experiments. This study was supported by a Grant-in-Aid from the Program for Changjiang Scholars and Innovative Research Teams in Universities (IRT0607) from China.

References

2. Chen CL. Inhibin and activin as paracrine/autocrine factors. Endocrinology 1993; 132: 4–5. [Medline] [CrossRef]
9. Bilezikjian LM, Blount AL, Donaldson CJ, Vale WW. Pituitary actions of ligands of
the TGF-beta family: activins and inhibins. Reproduction 2006; 132: 207–215. [Medline] [CrossRef]


24. Kenny HA, Woodruff TK. Follicle size class contributes to distinct secretion patterns of inhibin isoforms during the rat estrous cycle. Endocrinology 2006; 147: 51–60. [Medline] [CrossRef]


38. Young JM, McNellis AS. Inhibin removes the inhibitory effects of activin on steroid enzyme expression and androgen production by normal ovarian thecal cells. J Mol Endocrinol 2012; 48: 49–60. [Medline] [CrossRef]

