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Role of activin, inhibin, and follistatin in the pathogenesis of bovine cystic ovarian disease

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ABSTRACT

Cystic ovarian disease (COD) is an important cause of infertility in dairy cattle. Although many researchers have focused their work on the endocrine changes related to this disease, evidence indicates that intraovarian components play an important role in follicular persistence. Activin, inhibin, and follistatin participate as intraovarian regulatory molecules involved in follicular cell proliferation, differentiation, steroidogenesis, oocyte maturation, and corpus luteum function. Given the importance of these factors in folliculogenesis, we examined the expression and immunolocalization of activin/inhibin βA-subunit, inhibin α-subunit, and follistatin in the ovaries of healthy estrus-synchronized cows and in those of cows with spontaneous or adrenocorticotropic hormone (ACTH)-induced COD. We also studied inhibin B (α βB) levels in serum and follicular fluid. We found an increased expression of the βA-subunit of activin A/inhibin A, the α-subunit of inhibin, and follistatin in granulosa cells of spontaneous follicular cysts by immunohistochemistry, and decreased concentrations of inhibin B (α βB) in the follicular fluid of spontaneous follicular cysts. These results, together with those previously obtained, indicate that the expression of the components of the activin–inhibin–follistatin system is altered. This could lead to multiple alterations in important functions in the ovary like the balance between pro- and anti-apoptotic factors, follicular proliferation/apoptosis, and steroidogenesis, which may contribute to the follicular persistence and endocrine changes found in cattle with COD.

1. Introduction

Cystic ovarian disease (COD) is an important cause of infertility in dairy cattle and has been defined as the presence of one or more follicular structures in the ovaries, of at least 20 mm in diameter, which persist in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia et al., 2002; Peter, 2004; Vanholder et al., 2006). Many factors such as stress, nutritional management, and infectious diseases can cause COD in cattle. However, the primary cause of this disease has not yet been elucidated. It is accepted that the main component of the ethio-pathogenesis of COD is related to the hypothalamus–pituitary–ovarian axis. However, the

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persistence of the follicles in the absence of ovulation is related to an intraovarian component (Silvia et al., 2002).

Multiple intraovarian factors participate in the autocrine/paracrine signaling between theca interna cells, granulosa cells, and oocytes and contribute to a coordinated program of follicular cell proliferation and differentiation. Particularly in the later stages of follicular development, intraovarian factors also modulate the sensitivity of follicular cells to gonadotropins and other extraovarian factors (Glister et al., 2010). Locally produced regulatory factors include various members of the transforming growth factor beta (TGFB) superfamily, which in turn includes bone morphogenetic proteins, activins, and inhibins (Mihm and Austin, 2002).

Much evidence indicates that activins, follistatin and, to a lesser extent, inhibins synthesized by follicular cells exert local autocrine–paracrine actions to modulate follicular growth, gonadotropin responsiveness, steroidogenesis, oocyte maturation, ovulation, and corpus luteum function (Nishimori and Matzuk, 1996).

Inhibins and activins are composed of three subunits, α, βA, and βB, derived from three different precursor polypeptides encoded by distinct genes (Ying, 1988). The subunits combine to make the different forms of activins and inhibins. The dimerization of the β-subunits gives rise to three forms of activin referred to as activin A (βAβA), activin AB (βAβB), and activin B (βBβB). Dimers of an α-subunit and either a βA- or βB-subunit generate inhibin A (αβA) or inhibin B (α βB) (Knight, 1996; Knight and Glister, 2001, 2003, 2006). Follistatin is a cysteine-rich monomeric glycoprotein encoded by a single gene, structurally unrelated to the TGFB superfamily, but functionally linked through its role as a high-affinity binding protein for activins. Follistatin has several different isoforms due to alternative mRNA splicing and post-translational modifications (Knight, 1996; Knight and Glister, 2001, 2003, 2006).

The genes for both inhibin subunits are expressed in granulosa cells and affect follicle growth and steroidogenesis directly in vivo (Woodruff et al., 1990) and in vitro (Wrathall and Knight, 1995). Activin and inhibin have opposite effects, both at the pituitary and ovarian levels, because both compete for the type II activin receptor (Gray et al., 2001). Activin also enhances FSH receptor expression and inhibin synthesis by granulosa cells and promotes granulosa cell proliferation and steroidogenesis during early follicular development (Xiao et al., 1992). However, activin interaction with its receptors is regulated by follistatin levels. Follistatin specifically neutralizes activin functions in the pituitary and the ovary (Robertson et al., 1987) and has, therefore, inhibin-like activity.

Considering the importance of these factors in folliculogenesis, we hypothesized that an imbalance in the activin–inhibin–follistatin system may result in ovarian alterations such as follicular persistence that could contribute to the pathogenesis of COD. Therefore, in the present study, we examined the immunolocalization and expression of the activin/inhibin βA-subunit, inhibin α-subunit, and follistatin in the ovaries of healthy cows and animals with spontaneous or adrenocorticotropic hormone (ACTH)-induced COD. The concentration of inhibin B in follicular fluid (FF) and serum was measured in controls and animals with spontaneous COD.

2. Materials and methods

2.1. Induction and detection of cysts

All procedures were evaluated and approved by the Institutional Ethics and Security Committee (Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina; Protocol number: 44/10) and are consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010).

2.1.1. Induction of COD by adrenocorticotropic administration

Ten nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight; maintained under standard husbandry conditions) with regular estrous cycles were used. The stages of the estrous cycles were synchronized using the Ovsynch protocol: the animals were injected with a gonadotropin-releasing hormone (GnRH) analog (Buserelin acetate, Gonaxal®, Biogénesis-Bagó, Buenos Aires, Argentina, 10 μg/animal) on day 9, a Prostaglandin $F_{2\alpha}$ analog (D+ Cloprostenol, Enzaprost D-C®, Biogénesis-Bagó, Argentina, 150 μg/animal) on day 2, and a GnRH analog (Buserelin acetate, Gonaxal®, Biogénesis-Bagó, Buenos Aires, Argentina, 10 μg/animal) on day 0. The time of ovulation was monitored by transrectal ultrasonography and designated as day 1 of the estrous cycle, because ovulation occurs 24–32 h after the second injection of GnRH (Pursley et al., 1995).

The model of ACTH-induced ovarian follicular cysts used in the present study has been previously described and characterized (Dobson et al., 2000; Ortega et al., 2008; Salvetti et al., 2010; Amweg et al., 2013). Briefly, beginning on day 15 of a synchronized estrous cycle, five heifers received subcutaneous injections of 1 mg of a synthetic polypeptide with ACTH activity (Synacthen Depot, Novartis, Basel, Switzerland), every 12 h for 7 days (ACTH-treated group). The other five animals received saline solution (1 ml) (control group).

Ovarian ultrasonographic examinations were performed in all animals, using a real-time, B-mode scanner equipped with a 5 MHz, linear-array, transrectal transducer (Honda HS101V, Japan) (Sirois and Fortune, 1988). The growth and regression of follicles >5 mm, corpora lutea, and follicular cysts were monitored. Daily ovarian ultrasonography was performed throughout a complete estrous cycle in control heifers (21–23 days) and from day 14 (day 0 = day of ovulation) until ovariectomy approximately on day 48 in ACTH-treated heifers. Follicular cysts detected by ultrasonography were defined as any follicular structure with a diameter equal to or greater than 20 mm present for 10 days or more, without ovulation or corpus luteum formation (Dobson et al., 2000). The first day of follicular cyst formation was the day a follicle attained 20 mm or more in diameter and the ovaries were removed 10 days later by flank laparotomy (approximately day 48). In five heifers of the control group, ovariectomy was conducted

when the dominant follicle reached a diameter greater than 10 mm, in the absence of an active corpus luteum, to obtain pre-ovulatory follicles (approximately day 18).

Blood samples were obtained daily throughout the entire experiment to test the corresponding hormone in parallel studies (Ortega et al., 2008; Amweg et al., 2013).

2.1.2. Spontaneous COD

Argentinean Holstein cows from dairy herds of the milk-producing region of Santa Fe, Argentina, with spontaneous COD were used to obtain whole ovaries by ovariectomy or FF by follicular aspiration. Cows were diagnosed with the disease during the periodic reproductive control by rectal palpation and ultrasonography (B-mode ultrasound equipped with a transrectal 5.0 MHz linear-array transducer, HS-101V, Honda Co., Japan), considering previously described parameters (Dobson et al., 2000).

The ovaries from 10 animals were removed by transvaginal ovariectomy and blood samples were collected just before surgery for hormone analysis.

The FF from animals with COD ($n = 15$) and controls ($n = 10$) was aspirated using a digital ultrasound system 8300vet Chison equipped with a micro-convex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, Brazil). The FF was transported to the laboratory at around 4 °C temperature for processing and then stored at −20 °C until their use in the enzyme-linked immunosorbent assay (ELISA). Blood samples were collected before the procedure, processed, and stored for hormone analysis.

2.2. Tissue sampling and follicular classification

To prevent the rupture of the follicular cysts during surgery, the FF was aspirated before ovariectomy. The FF was transported to the laboratory at 4 °C for processing and then stored at −20 °C until use. Small samples from ovarian tissues of each group were immediately frozen at −80 °C until their use in western blotting to determine the specificity of the antibodies used in immunohistochemistry and to evaluate follistatin isoforms. The health status of the follicles was confirmed by hormonal concentrations in FF (Ortega et al., 2008; Amweg et al., 2013).

The ovaries were fixed in 4% buffered formaldehyde for 8–10 h at 25 °C and then washed in phosphate-buffered saline (PBS). Then, fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and primarily stained with hematoxylin–eosin for a preliminary observation of the ovarian structures (Salvetti et al., 2009). Follicles were classified into the following groups: primary, secondary, tertiary, atretic follicles (Priedkalns, 1998), and follicular cysts (Silvia et al., 2002). Only follicular cysts with a complete granulosa cell layer were analyzed.

2.3. Immunohistochemistry

The details, suppliers and concentrations of the antibodies used are reported in Table 1. Each antibody was assayed in five sections (minimum) of each ovary from each animal, a total of approximately 15 sections were taken for immunohistochemical quantification from each animal. A streptavidin–biotin immunoperoxidase method was performed as previously described (Salvetti et al., 2010). Briefly, after deparaffinization, microwave pretreatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0). The endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and nonspecific binding was blocked with 10% (v/v) normal goat serum. All sections were incubated with the primary antibodies for 18 h at 4 °C and then for 30 min at room temperature with biotinylated secondary antibodies (CytoScan™ HRP Detection System). The antigens were visualized by the CytoScan™ HRP Detection System (Cell Marque, Rocklin, CA, USA), and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Invitrogen, Camarillo, CA, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's hematoxylin, dehydrated, and mounted.

To verify immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit, and mouse nonimmune sera. The specificity of the secondary antibodies was tested by incubation with primary antibodies raised against human antigens with a proven negative reaction to tissues of cattle: anti-CD45 (Clone: PD7/26; Dako, Carpinteria, CA, USA) and anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA). To exclude the possibility of nonsuppressed endogenous peroxidase activity, some sections were incubated with DAB alone.

2.4. Western blotting

To test the specificity of the antibodies that detect the activin/inhibin βA-subunit and the inhibin α-subunit and to evaluate follistatin isoforms, complete walls of tertiary follicles and follicular cysts were homogenized in a radioimmunoprecipitation assay lysis buffer (RIPA) consisting of 1% (v/v) IGEPAL CA630 (octylphenyl-polyethylene glycol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma-Aldrich Corp., New York, USA), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Follicle homogenates were centrifuged at 14,000 × *g* for 20 min and the supernatant was stored frozen at −80 °C. Proteins (40 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15% resolving gel), transferred onto nitrocellulose membranes (Amersham-GE Healthcare, Buckinghamshire, UK), blocked for 1 h in 2% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (Sigma-Aldrich Corp., NY, USA), and then incubated overnight at 4 °C with specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with the corresponding secondary

Table 1
Antibodies, suppliers, and dilutions used for immunohistochemistry (IHC) and western blotting (WB).

Antibodies	Clone/source	Dilution	
		IHC	WB
Primary antibodies			
Anti-activin A	Mouse anti-activin beta-A. Clone E4 (Serotec, Oxford, UK)	1:50	1:100
Anti-inhibin	Mouse anti-human inhibin alpha. Clone R1 (Serotec, Oxford, UK)	1:75	1:100
Anti-follistatin	Rabbit policlonal for follistatin. Rab64490 (Abcam, Cambridge, MA, USA)	1:100	1:100
Secondary antibodies			
Biotinylated link	CytoScan Biotinylated Link, CytoScan™ HRP Detection System (Cell Marque, CA, USA)	Ready to use	–
HRP-anti-mouse IgG	Goat Polyclonal. sc-2005 (Santa Cruz Biotechnology, Inc., CA, USA)	–	1:2000
HRP-anti-rabbit IgG	Goat Polyclonal. sc-2004 (Santa Cruz Biotechnology, Inc., CA, USA)	–	1:2000

peroxidase-conjugated antibody (Table 1). The immunopositive bands were detected by chemiluminescence, using the ECL-plus system (Amersham-GE-Healthcare, Buckinghamshire, UK) on hyperfilm-ECL film (Amersham-GE-Healthcare, Buckinghamshire, UK).

2.5. Inhibin B (α βB) concentration in follicular fluid and serum

Serum and FF concentrations of inhibin-B (α βB) in control animals and animals with spontaneous COD were quantified using a commercial ELISA kit (Inhibin-B Gen II, Beckman Coulter, CA, USA), following the manufacturer's instructions. Initially, all FF samples were diluted 10-fold in fetal bovine serum. FF samples, serum (undiluted), and standards were placed in duplicate in the wells of the plate and then placed in the specific test buffer provided and incubated at room temperature for about 2 h. Samples were washed and biotin-conjugated antibody was added and incubated for 1 h. Finally, after appropriate washings, streptavidin conjugated to horseradish peroxidase was added and incubation was performed for about 30 min. The reaction was evidenced by the addition of 3,3′,5,5′-tetramethylbenzidine as chromogen solution, which was incubated for 15 min. The reaction was stopped with H_2SO_4 and readings were made at 450 nm with an ELISA reader (MS Microplate Reader, Thermo/Labsystems Inc., IL, USA). The detection limit of the kit is 2 pg/ml, and the coefficients of intra- and interassay variation were 8 and 4%, respectively.

2.6. Image analysis

Images were analyzed using Image Pro-Plus 3.0.1 (Media Cybernetics, Silver Spring, MA, USA).

For immunohistochemistry, images were digitized using a CCD color video camera (Motic 2000, Motic China Group, China) mounted on a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40×. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections in which the primary antibodies were replaced with rabbit and mouse non-immune sera. The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method (Ranefall et al., 1998). The image analysis score was calculated separately in each follicular wall layer (granulosa and theca interna) from at least 50 images of the secondary, tertiary, atretic, and cystic follicles from ovaries of all groups. The slides were scanned left to right from the top and all follicles in the selected categories (between 8 and 15 in the sections evaluated) were analyzed. For secondary follicles, all images covering granulosa and theca cells within each follicle were analyzed. For each tertiary, atretic and cystic follicle, 50 images/compartment (granulosa or theca interna)/follicle were quantified.

The percentage of the immunohistochemical stained area (IHSA) was calculated as a percentage of the total area evaluated through the color segmentation analysis, which extracts objects by locating all objects of the specific color (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5) and a mask was then applied to separate the colors permanently. The images were then transformed to a bi-level scale TIFF format. The methodological details of image analysis as a valid method for quantification have been described previously (Ortega et al., 2009; Salvetti et al., 2010).

For the western blot, the exposed films were scanned at 1200 dpi (scanner HP Officejet all in one J5780). The level of each isoform of follistatin in homogenates of small, medium, and large tertiary follicles and follicular cysts was analyzed by densitometry to obtain an integrated optical density value. This value was compared between samples from healthy and cystic ovaries and the relative expression was determined using actin as the loading control (Velázquez et al., 2010).

2.7. Statistics

The adequate number of images per follicle and the number of follicles per category were confirmed from a sample size calculation that evaluated the number of samples necessary to produce an estimate of the immunoreactivity that would fall within 0.4 units of the real value. SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to perform the statistical tests. The differences between the groups of data were assessed by

one-way ANOVA, followed by Duncan's multiple range tests. The distribution of data was tested for normality using the Kolmogorov–Smirnov test and the differences between two groups were detected by non-paired two-tailed Student's *t*-test. $P < 0.05$ values were considered significant. Results are expressed as mean ± standard deviation (SD).

3. Results

3.1. Experimental model

Successful induction of COD by ACTH was confirmed by the ovarian morphology and hormone concentrations. In ACTH-treated animals, healthy developing follicles, follicles showing different degrees of atresia, and one large follicular cyst (in one ovary) with a complete granulosa cell layer were observed. An active corpus luteum was absent in all cases. Ovaries from estrus-synchronized control animals exhibited follicles in various stages of development including primary, secondary, and tertiary follicles, as well as atretic follicles and regressed corpora lutea. In addition, the induction was confirmed by serum and FF hormone analysis (Amweg et al., 2013). Spontaneous COD was also confirmed by ovarian morphology and hormone concentrations (Amweg et al., 2013).

3.2. Antibody specificity

The results from western blot analyses of ovarian homogenates are summarized in Figs. 1, 2 and 4. Western blot analysis detected intense positive bands of appropriate sizes for each of the molecules studied by immunohistochemistry (activin/inhibin βA-subunit, inhibin α-subunit, and follistatin). The activin βA-subunit was detected as a single band at 58 kDa, the inhibin α-subunit as a single band at 32 kDa, and follistatin as multiple bands at 31, 35, 37, 41, and 65 kDa corresponding to the different isoforms, which were quantified differentially.

3.3. Immunolocalization and expression of activin A/inhibin A (βA-subunit)

To obtain information regarding the localization of activin A/inhibin A (βA-subunit) in different follicular structures, their expression was evaluated by immunohistochemistry and semi-quantitative image analysis. The protein was localized in the cytoplasm of granulosa, and theca interna cells of all follicles analyzed. In estrus-synchronized control animals, a moderate expression of the activin βA-subunit was observed in granulosa cells of primary and secondary follicles without differences in ACTH-induced and spontaneous cysts. However, the re were differences in the same categories between ACTH-induced and spontaneous cysts, the latter having the highest expression ($P < 0.05$) (Fig. 1). No differences were found between groups for tertiary and atretic follicles. When comparing follicular cysts from cows with spontaneous and induced COD with tertiary and atretic follicles from the control group, the highest expression was found in both induced and spontaneous cystic follicles ($P < 0.05$). The immunopositive area in the theca interna was lower than that in granulosa cells ($P < 0.05$) and no differences were found in secondary and tertiary follicles for all groups. Atretic follicles from the control group showed the lowest expression regarding tertiary follicles from the group with spontaneous cysts ($P < 0.05$).

3.4. Inhibin

3.4.1. Immunolocalization and expression of the inhibin α-subunit

Inhibin (α-subunit) was expressed only in granulosa cells of large tertiary follicles in all three groups and in both induced and spontaneous cysts (Fig. 2A and C). The tertiary follicles from the different groups showed no differences, but increased immunostaining for inhibin α-subunit was observed in spontaneous follicular cysts compared with tertiary follicles from any group and with cystic follicles from the ACTH-treated group ($P < 0.05$).

3.4.2. Inhibin B (α βB) serum and follicular fluid concentration

The analysis of serum and FF concentration of inhibin B showed no differences in serum concentration between control and cystic animals, but a significantly higher FF concentration in healthy tertiary follicles than in cysts ($P < 0.05$) (Fig. 2B).

3.5. Follistatin

3.5.1. Immunolocalization and expression of follistatin in situ

Follistatin immunostaining was observed in the cytoplasm of granulosa and theca interna cells (Fig. 3). Ovaries from cows with spontaneous COD showed an increase in staining of all their follicles (in different stages of development) in relation to the other groups. Spontaneous cysts also showed higher staining than the tertiary and atretic follicles from the control group.

3.5.2. Differential follistatin isoform expression in complete follicular walls

Western blotting allowed detection and evaluation of follistatin isoforms corresponding to 31, 35, 37, 41, and 65 kDa. No significant differences were found between small, medium, and large tertiary follicles from the control group and cystic follicles (Fig. 4).

3.6. Expression pattern of the activin βA-subunit, inhibin α-subunit and follistatin at different follicular stages within each group

Comparison between the different follicular stages in the estrus-synchronized control group revealed a significant increase in activin βA-subunit expression in granulosa cells of tertiary follicles when compared with primary and secondary follicles and a moderate increase in atretic follicles ($P < 0.05$). The expression of the activin βA-subunit in the animals with ACTH-induced and spontaneous COD showed a similar pattern, with the highest expression in follicular cysts (Figs. 1B and 5).

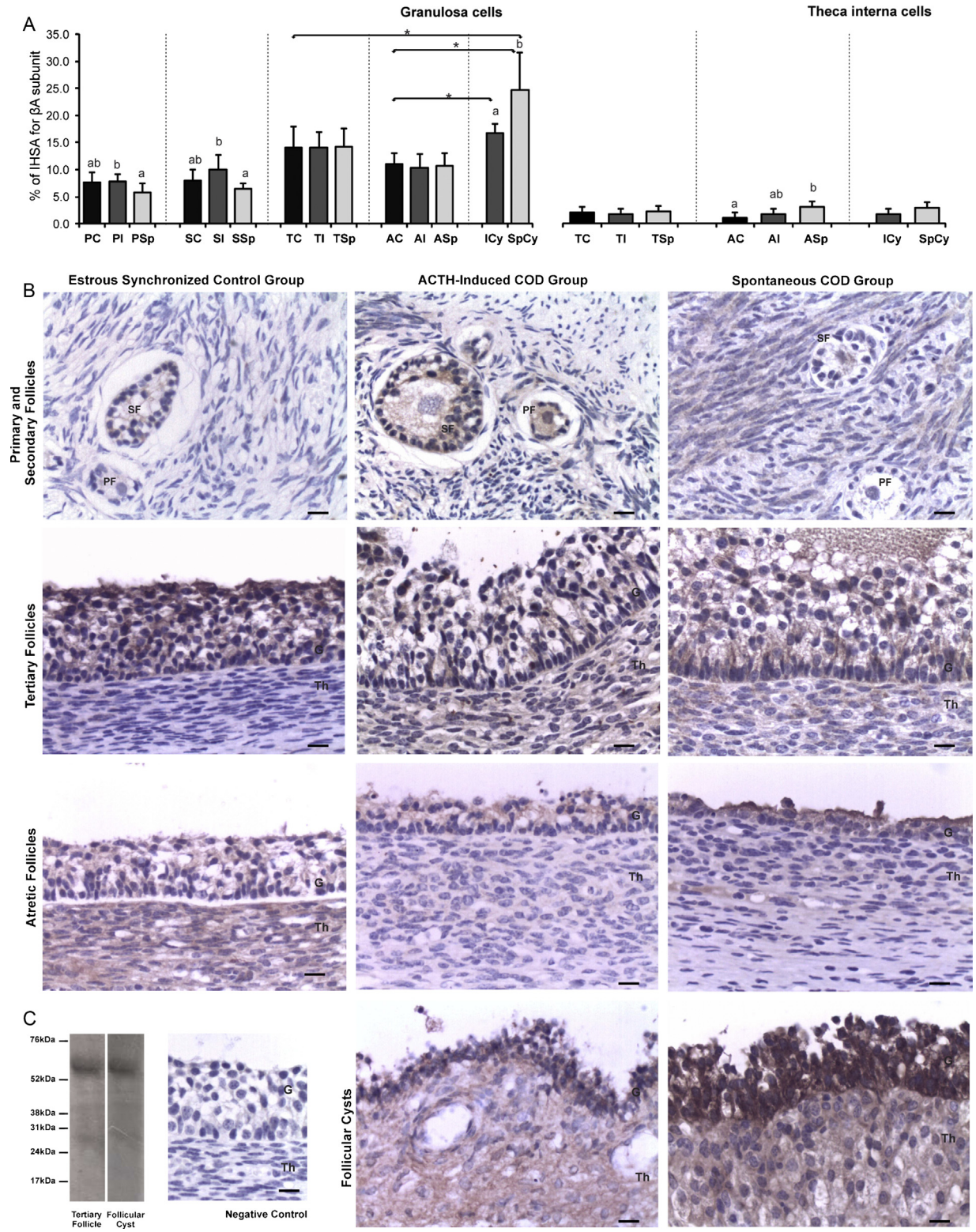

Fig. 1. Activin A expression in different follicular categories from the control, ACTH-induced COD, and spontaneous COD groups. (A) Relative expression (measured as percentage of immunopositive area) of Activin A (βA-subunit) in granulosa and theca interna cells of primary (P), secondary (S), tertiary (T), and atretic (A) follicles from the control group (C), ACTH induced-COD group (I), and spontaneous-COD group (Sp); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different ($P < 0.05$). Differences of activin expression in induced or spontaneous cystic follicles related to control tertiary or atretic follicles are also indicated by an asterisk ($P < 0.05$). (B)

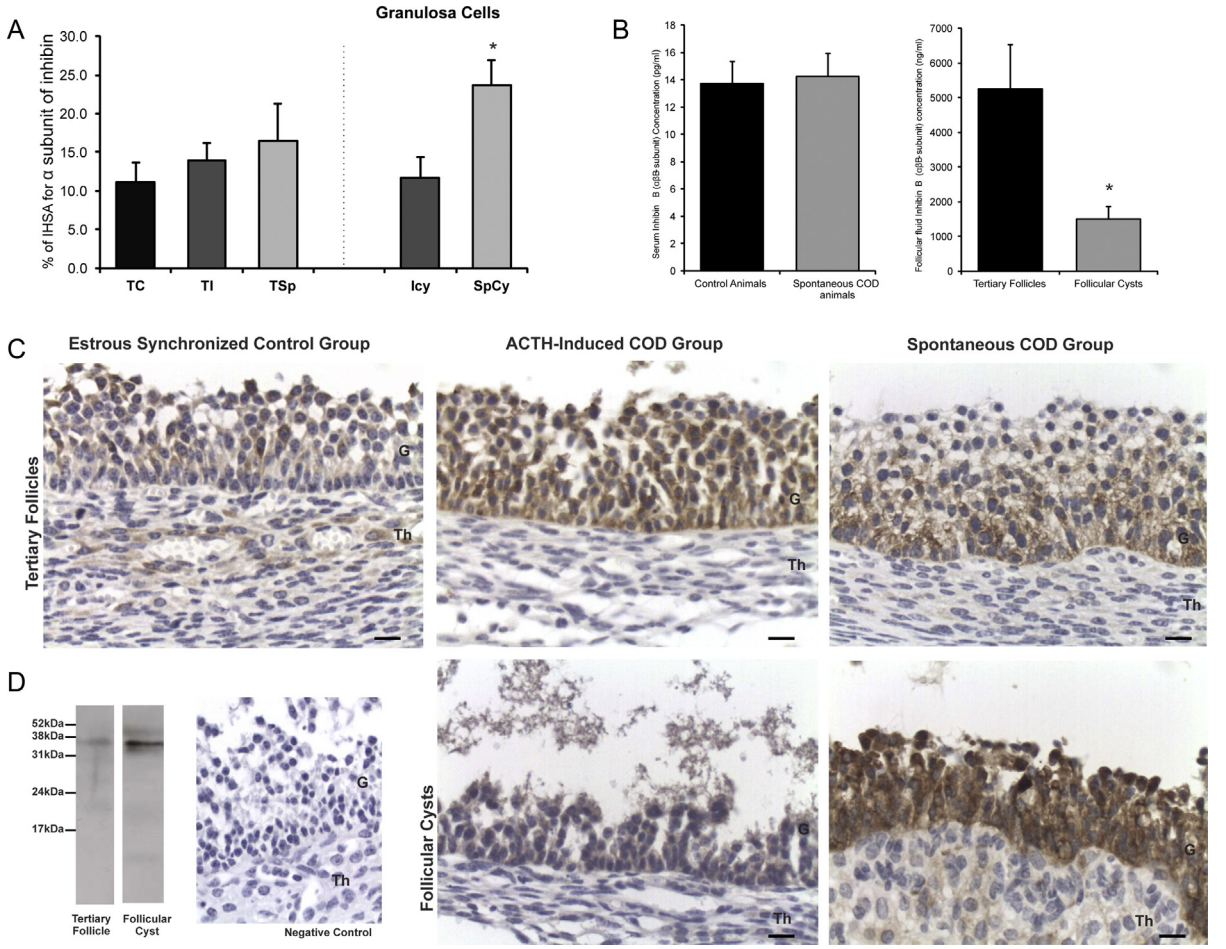

Fig. 2. Inhibin expression in different follicular categories from the control, ACTH-induced COD, and spontaneous COD groups. (A) Relative expression (measured as percentage of immunopositive area) of inhibin (α-subunit) in granulosa of tertiary (T) follicles from the control group (C), ACTH-induced COD group (I), and spontaneous COD group (Sp); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different ($P < 0.05$). Differences in inhibin expression in induced or spontaneous cystic follicles related to control tertiary follicles are also indicated by an asterisk ($P < 0.05$). (B) Inhibin B concentration in serum and FF of tertiary follicles from the estrus-synchronized control group and follicular cysts from the spontaneous COD group. Values represent mean ± SD. Bars with asterisk are significantly different ($P < 0.05$). (C) Representative images of inhibin immunostaining in tertiary follicles of the estrus-synchronized control group and cystic follicles of the spontaneous and ACTH-induced COD groups. Granulosa (G), Theca Interna (Th). Bars = 20 μm. (D) Verification of antibody specificity by western blotting analyses of ovarian homogenates and negative control of immunohistochemistry.

The animals with ACTH-induced COD showed no differences in inhibin α-subunit expression in granulosa cells between tertiary follicles and cysts. However, the expression of the inhibin α-subunit in the animals with spontaneous COD was higher in cysts than in tertiary follicles ($P < 0.05$) (Figs. 2B and 5).

Follistatin expression was increased throughout follicular development, with the highest expression observed in the tertiary follicles in control and ACTH-induced COD group and in cysts from the spontaneous COD group ($P < 0.05$) (Figs. 3B and 5).

The expression of all the molecules studied was significantly lower in theca cells than in granulosa cells in all follicular categories, except for the inhibin α-subunit, whose expression was not evident in theca cells.

4. Discussion

In the present study, we found an altered expression of the components of the activin–inhibin–follistatin system in the ovaries of animals with COD. We found an increased expression of the βA-subunit, corresponding to activin A/inhibin A, and the α-subunit of inhibin in the ovaries of animals with spontaneous COD. The β-subunits share a high sequence identity (63%), but are differentially regulated during development and throughout ovarian

Representative images of activin A immunostaining in primary, secondary, tertiary, and atretic follicles of the estrus-synchronized control group and cystic follicles of spontaneous and ACTH-induced COD groups. Granulosa (G), Theca Interna (Th). Bars = 20 μm. (C) Verification of antibody specificity by western blotting analysis of ovarian homogenates and negative control of immunohistochemistry.

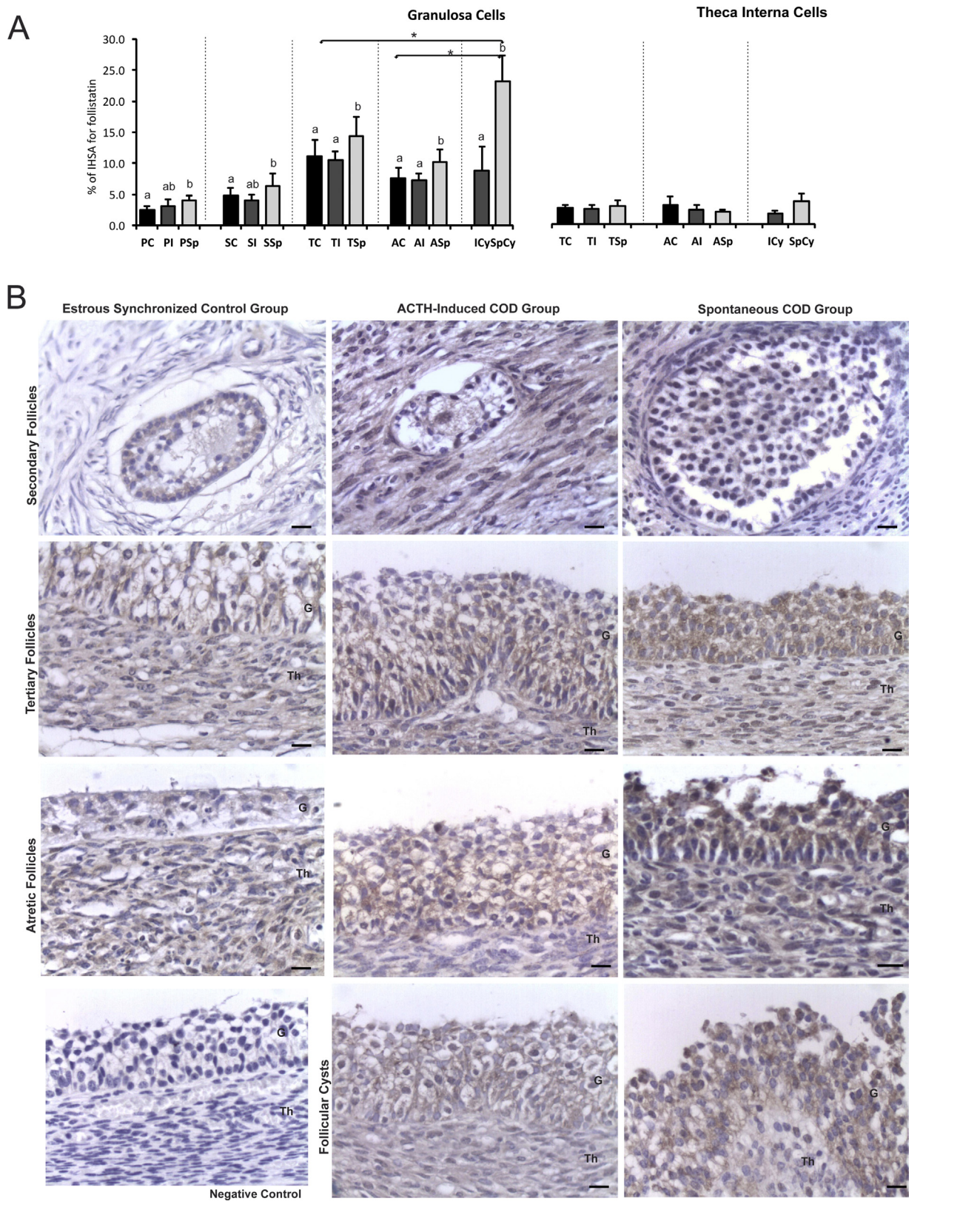

Fig. 3. Follistatin expression in different follicular categories from the control, ACTH-induced COD, and spontaneous COD groups. (A) Relative expression (measured as percentage of immunopositive area) of follistatin in granulosa and theca interna cells of primary (P), secondary (S), tertiary (T), and atretic (A)

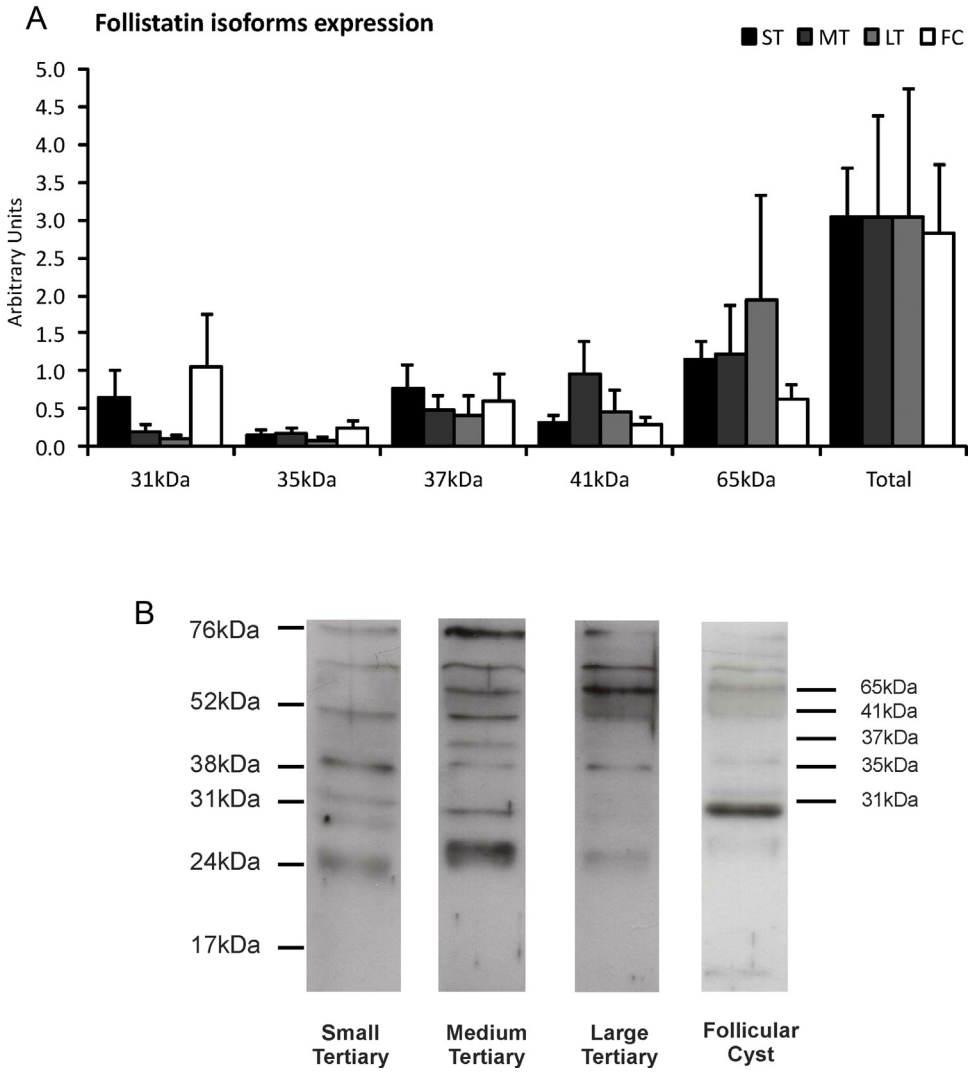

Fig. 4. Expression of each follistatin isoform in complete follicular wall from different follicular categories. (A) Relationship between follicular category and the relative abundance of the five different follistatin isoforms in complete follicular wall (arbitrary units). The apparent molecular weight values of the five isoforms were 65, 41, 37, 35, 31 kDa. Values are expressed as mean ± SD. (B) Representative immunoblot showing the presence of the five different follistatin isoforms in small, medium, and large control tertiary follicles as well as in follicular cysts. The positions of the marker proteins used to calibrate the blots are indicated (left) as the apparent molecular weight values of the follistatin bands identified (right).

follicle selection and maturation (Muttukrishna et al., 1994). Expression differences are reflected in functional differences (Thompson et al., 2004). Various studies have shown that the expression of the subunits that form inhibin and activin is not consistent between species. For example, in contrast to that observed in cattle, Magoffin and Jakimiuk (1997, 1998) found high levels of inhibin A and B in FF from patients with polycystic ovarian disease. These authors showed that inhibin B is the predominant form of inhibin in the FF of polycystic ovaries as it is in normal ovaries. The concentration of inhibin B was 10 times higher than that of inhibin A and 50 times higher than that of activin A (Magoffin and Jakimiuk, 1997, 1998).

Our results indicate that the high levels of expression of total inhibin α-subunit in tissue sections probably correspond mostly to inhibin βA, considering that intrafollicular levels of inhibin βB in cysts were lower than those found in large tertiary follicles in the control group. Furthermore, the antibody used in the immunohistochemistry technique for activin localization detects exclusively the βA-subunit,

follicles from the control group (C), ACTH-induced COD group (I), and spontaneous COD group (Sp); ACTH-induced cysts (ICy) and spontaneous cysts (SpCy). Values represent mean ± SD. Bars with different letters within a category are significantly different ($P < 0.05$). Differences in follistatin expression in induced or spontaneous cystic follicles related to control tertiary or atretic follicles are also indicated by an asterisk ($P < 0.05$). (B) Representative images of follistatin immunostaining in primary, secondary, tertiary, and atretic follicles of the estrus-synchronized control group and cystic follicles of the spontaneous and ACTH-induced COD groups. A negative control of immunohistochemistry is shown. Granulosa (G), Theca Interna (Th). Bars = 20 μm.

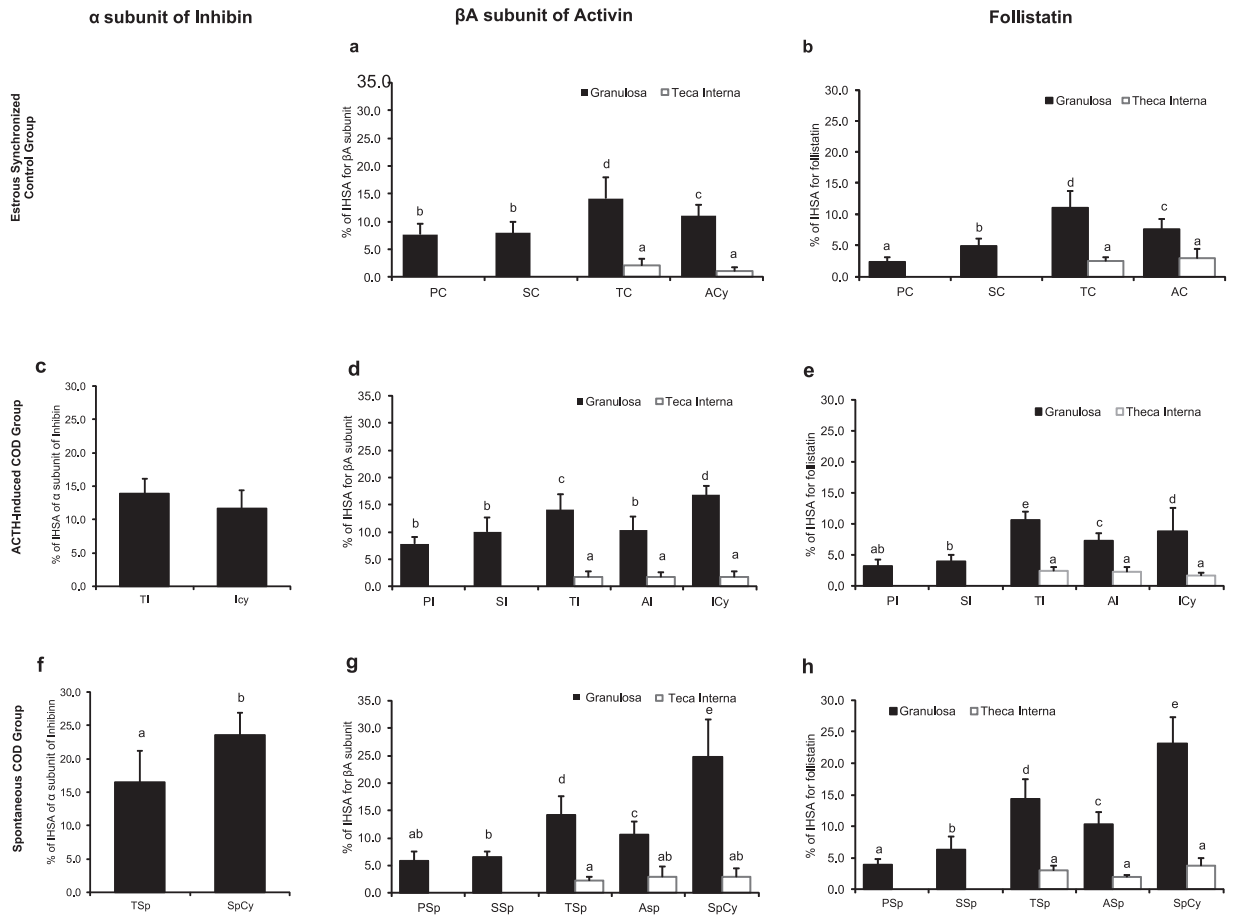

Fig. 5. Relative expression (measured as percentage of immunopositive area) of inhibin, activin, and follistatin in granulosa and theca interna cells of primary, secondary, tertiary, and atretic follicles (all groups) and cystic follicles (ACTH-induced and spontaneous COD groups) within each group. Values represent mean ± SD. Bars with different letters are different ($P < 0.05$).

which is shared between activin βA and inhibin βA. In large antral follicles of cows, Beg et al. (2002) found that inhibin βB concentration decreases between the 8.0- and 8.9- and between the 10.0- and 10.9-mm follicular diameter and that activin A increases between the 7.0- and 7.9- and between the 9.0- and 9.9-mm follicular diameter ranges. The inhibin B concentration observed by Beg et al. (2002) in healthy cows coincides with that found by us in FF of large follicles in control animals. The decrease in inhibin B concentration observed in the cysts could be related to a lower expression of the βB-subunit and to a higher expression of the βA-subunit, which leads the equilibrium to the greater expression of activin A/inbibin A found.

In cystic cows, Kaneko et al. (2002) demonstrated that plasma inhibin A remained at high levels for a long period, associated with growth of persistent dominant follicles, and then declined as dominant follicles either ceased to grow or ovulated. However, these authors did not measure the concentration of follicular inhibin A. Beg et al. (2002) found similar levels of total and inhibin A in the follicles of different follicular waves in cows.

Many reports have confirmed the autocrine/paracrine effects of activin to promote proliferation and maintain granulosa cell differentiation (Knight et al., 2012). For instance, treatment of granulosa cells from diethylstilbestrol (DES)-treated immature rats with activin alone can up-regulate FSH receptor expression, enhance responsiveness to FSH and luteinizing hormone, and increased expression of inhibin α-subunit and follistatin mRNAs (Knight, 1996). In the presence of FSH, activin can enhance aromatase activity/estrogen production and progesterone secretion as well as increasing the expression of FSH receptor and follistatin (Knight, 1996). This widely accepted action of activin in promoting FSH receptor expression would operate most successfully at low follistatin levels (Knight, 1996). Activin A also reduces androgen production (Hillier et al., 1991), while activin B suppresses androgen production from theca cells cultured in vitro (Young et al., 2012).

Although in the present study we found increased expression of the activin βA-subunit, this is not totally related to changes typically seen in follicular cysts such as a decrease in proliferation and apoptosis (Salvetti et al., 2010) and increased levels of androgens and normal concentrations of estrogen (Amweg et al., 2013). Previous studies have shown that the balance of pro- and anti-apoptotic proteins is in favor of cell survival, but with low levels of cell proliferation (Isobe and Yoshimura, 2007;

Salvetti et al., 2009, 2010). However, due to the interaction between the system's components, the results regarding the expression of inhibin and follistatin and the relationship between these molecules must also be considered.

Activins and inhibins, secreted by granulosa cells, can function as reciprocal regulators of the androgen production of theca cells. As inhibin production by the selected follicle increases, the inhibitory action of activin on androgen production is overcome and the follicle produces increasing amounts of androgen, vital to maintain sufficient substrate for aromatization to estrogen (Knight et al., 2012). The levels of the inhibin α-subunit and the inhibin βA-subunit found in follicular cysts are assumed to functionally exceed activin βA levels, resulting in a balance tilted in favor of the actions of inhibin in the ovary.

Follistatin binds to activin isoforms and, to a lesser extent, to inhibin isoforms through their common β-subunit. This action is considered to represent the main functional role of follistatin, particularly with regard to the neutralization of activin bioactivity since the affinity of follistatin for activin is similar to that between activin and its receptors (Knight, 1996). In the current study, the increases in follistatin levels coincide with increases in the size of the follicles, with the highest levels of expression found in the tertiary follicles (in all groups) and cystic follicles. Moreover, follistatin levels in all follicles studied were higher in animals with spontaneous COD than in controls, unlike that observed in animals with ACTH-induced COD. However, when follistatin isoforms were assessed in complete follicular wall by western blotting, no difference was observed even for total follistatin. This could be because complete follicular wall samples, composed of granulosa and theca cell layers, were used and can be masking potential changes in specific isoforms. By opposing the positive actions of activin on granulosa cell, follistatin may promote follicle atresia associated with decreased aromatase activity, inhibin secretion, and increased progesterone production.

Based on our results, we could postulate that the delicate balance between the components of the inhibin–activin–follistatin system is altered in favor of a low tone of activin. This might contribute to the mechanisms that lead to prolonged follicular persistence, with low levels of apoptosis and proliferation in these structures (Isobe and Yoshimura, 2007; Salvetti et al., 2010) and with an altered steroidogenesis (Amweg et al., 2013).

Finally, the differences found between spontaneous and ACTH-induced COD are probably due to the differences in the environmental conditions and the time of persistence of the cysts, which could influence the expression of these components.

5. Conclusion

In summary, the results obtained in this study, together with previous results, indicate that there is an imbalance in the expression of components of the activin–inhibin–follistatin system that could contribute to follicular persistence and endocrine changes, thus contributing to the pathogenesis of bovine cystic ovarian disease.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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